From Department of Oncology & Pathology
Karolinska Institutet, Stockholm, Sweden

THE ROLE OF AUTOPHAGY IN ANTICANCER THERAPY

Matheus Dyczynski

Karolinska Institutet

Stockholm 2018

Published by Karolinska Institutet.
Printed by E-Print AB 2018

ISBN 978-91-7831-182-8

THE ROLE OF AUTOPHAGY IN ANTICANCER THERAPY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Matheus Dyczynski

Principal Supervisor:
Katja Pokrovskaja Tamm
Karolinska Institutet
Department of Oncology & Pathology

Co-supervisor(s):
Angelo De Milito
Karolinska Institutet
Department of Oncology & Pathology

Opponent:
Margrét Helga Ögmundsdóttir
University of Iceland
Department of Biochemistry & Molecular Biology

Examination Board:
Nico Dantuma
Karolinska Institutet
Department of Cell & Molecular Biology

Karin Öllinger
Linköping University
Department of Clinical & Experimental Medicine
Division of Cell Biology

Vladimir Bykov
Karolinska Institutet
Department of Oncology & Pathology

To Katja & Danne
who made this possible

'Non mors timenda, sed vita non incohata!'
"It is not death that one should fear, but one should fear never beginning to live!"
Probably not Marcus Aurelius.

LIST OF SCIENTIFIC PAPERS

I. Pellegrini P, **Dyczynski M**, Sbrana FV, Karlgren M, Buoncervello M, Hägg-Olofsson M, Ma R, Hartman J, Bajalica-Lagercrantz S, Grandér D, Kharaziha P, De Milito A. Tumor acidosis enhances cytotoxic effects and autophagy inhibition by salinomycin on cancer cell lines and cancer stem cells. *Oncotarget.* 2016; 7(24):35703-35723.

II. **Dyczynski M**, Vesterlund M, Björklund AC, Zachariadis V, Janssen J, Gallart-Ayala H, Daskalakis E, Wheelock CE, Lehtiö J, Grandér D, Pokrovskaja Tamm K, Nilsson R. Metabolic reprogramming of acute lymphoblastic leukemia cells in response to glucocorticoid treatment. *Cell Death & Disease.* 2018, in press

III. **Dyczynski M**, Yu Y, Otrocka M, Parpal S, Braga T, Henley AB, Zazzi H, Lerner M, Wennerberg K, Viklund J, Martinsson J, Grandér D, De Milito A, Pokrovskaja Tamm K. Autophagy inhibition by small molecule inhibitors of Vps34 improves sensitivity of breast cancer cells to Sunitinib. *Cancer letters*. 2018; 435:32–43.

IV. **Dyczynski M**, Knopf P, Serviss J, Lerner M, Grandér D, Pokrovskaja Tamm K. Regulation of RASAL2 and RASAL2-AS during autophagy. *Manuscript*.

PAPERS NOT INCLUDED IN THE THESIS

I. Ramachandran M, Yu D, **Dyczynski M**, Baskaran S, Zhang L, Saul S, Lulla A, Lulla V, Nelander S, Dimberg A, et al. Safe and effective treatment of experimental neuroblastoma and glioblastoma using systemically administered triple microRNA-detargeted oncolytic Semliki Forest virus. *Clinical Cancer Research*. 2016.

II. Kolosenko I, Yu Y, Busker S, **Dyczynski M**, Liu J, Haraldsson M, Palm Apergi C, Helleday T, Tamm KP, Page BDG, et al. Identification of novel small molecules that inhibit STAT3-dependent transcription and function. *PLoS ONE*. 2017; 12:e0178844.

III. Review: Onorati AV, **Dyczynski M**, Ojha R, Amaravadi RK. Targeting autophagy in cancer. *Cancer* 2018; 466:68.

ABSTRACT

Autophagy is a fundamental catabolic process, which is utilized by nearly every cell and tissue type upon stress exposure and has been shown to contribute to resistance to chemotherapy in a variety of cancers. The subject of this thesis is to shed light on the role of autophagy in chemotherapy and to investigate novel regulators of autophagy.

Multiple clinical trials have been started in order to overcome resistance to standard therapy by combining it with lysosomal inhibitor hydroxychloroquine, yet with limited success. This drug has been shown to have poor cell uptake properties in solid tumors due to tumor acidosis. In **paper I** we found that the compound Salinomycin is a potent autophagy inhibitor in multiple cancer cell lines, especially under acidic conditions. Salinomycin was able to penetrate the acidic core of multicellular spheroids and decrease cell viability and clonogenic survival of colorectal cancer cells. We also show that Salinomycin efficiently blocked autophagic flux in breast cancer cells. In particular, cancer stem cells derived from cell lines or primary breast cancer tumors showed reduced viability and reduced capability to form mammospheres under Salinomycin treatment. Using mass spectrometry, we could confirm pH-dependent intracellular accumulation of Salinomycin. This data proves the potency of Salinomycin as an anti-cancer drug with capacities to modulate autophagy in the acidic tumor microenvironment.

Part of the standard treatment regimen of pediatric patients with Acute Lymphoblastic Leukemia (ALL) are glucocorticoids (GC). This metabolic hormone is effective in inducing cell death in ALL cells. GC mediated inhibition of glucose uptake and upregulation of catabolic processes such as autophagy have previously been reported. In **paper II** we addressed in detail what metabolic changes occur upon GC treatment in ALL cell lines by parallel time-course proteomics, metabolomics and isotope tracing, and by confirming selected findings by cross-referencing with publicly available microarray data and experimentally by qRT-PCR. Our findings confirmed the onset of growth arrest, autophagy and apoptosis. Not only glucose but also glutamine entry into the Citric-Acid-Cycle was inhibited contrasting the upregulation of glutamine-ammonia-ligase (GLUL) expression suggesting the induction of glutamine synthesis. Potentiating the GLUL-mediated reaction rescued cell viability and reduced autophagic flux suggesting that GLUL induction and glutamine synthesis are relevant for the autophagy induction and sensitivity of ALL cells to GCs. This data provides a comprehensive overview of metabolic changes in ALL cells upon GCs' treatment and may shed light on the mechanism of GC-induced cell death in ALL cells.

In **paper III** we used high-content microscopy to screen the FIMM drug library consisting of 306 anticancer drugs and identified 104 autophagy modulators, of which 16 showed cell death potentiation upon siRNA mediated knock-down of ATG7 (autophagy-related protein 7) and VPS34 (vacuolar protein sorting 34), key regulators of autophagy. We validated the hits in 2 breast cancer cell lines, MDA-MB231 and MCF7, and continued to characterize two of the hits, Erlotinib and Sunitinib, in detail. The collaboration with Sprint Bioscience led to the development of SB02024, a specific inhibitor of the VPS34 kinase. We showed that SB02024 could block autophagy in vitro and in in vivo xenograft mouse models. Combination of SB02024 with Sunitinib and Erlotinib increased cytotoxicity by these drugs in either 2D cell culture, colony formation assays, or, in case of Sunitinib, in cells grown in 3D as multicellular spheroids. This data further strengthens the notion that using VPS34 inhibitors in combination with targeted tyrosine kinase inhibitor-based therapy, and particularly Sunitinib, can overcome resistance and emphasizes their value in cancer treatment.

RAS protein activator like 2 (RASAL2) is a known tumor-suppressor regulating members of the RAS-family of oncoproteins. In **paper IV** we describe for the first time a role for RASAL2 in the induction of autophagy. We found that autophagy induction via pharmaceutical mTOR inhibition or amino acid-starvation increased RASAL2 transcription. Furthermore, RASAL2 protein levels were regulated by autophagy-dependent protein degradation. Thus, in the starved cells, RASAL2 mRNA levels were induced while protein levels declined. Also, depletion of autophagy-related protein 7 (ATG7) that impaired autophagy process resulted in a striking increase in RASAL2 protein levels. RNAi-mediated knockdown of RASAL2 inhibited LC3-II accumulation or GFP-LC3 puncta formation. In silico analysis of RASAL2 revealed two potential LC3 interacting region motifs (LIR), which could point to an interaction between these two proteins. These data suggest that RASAL2 is involved in autophagy and is regulated by autophagy in a negative feedback manner.

PREAMBLE

One stands in awe of the complexity of life: billions of years of evolution have shaped archaea as well as pro- and eukaryotes in their ability to survive in the most diverse habitats. Life has evolved to thrive in hostile environments, from icy landscapes in the Arctic to scorching volcanic sulphur vents deep in the ocean, continually adapting to the conditions of the surroundings. Large intertwined networks carefully shaped by evolution to maintain homeostasis make this adaption possible while being flexible enough to change if necessary. The central dogma of molecular biology is at its heart, describing the residue-by-residue transfer of sequential information from DNA to RNA to protein. Researchers have identified transcriptional/translational regulation and modifications, positive and negative feedback loops, epigenetics, non-coding RNA and many other mechanisms, which enable the cells to react on internal and external stimuli, whilst keeping a delicate balance. However, what happens when this equilibrium is being tipped over?

We have extended our life expectancy by a great deal due to science and technology by limiting our exposure to life-threatening conditions such as malnutrition and pathogens. We have tremendously progressed in our understanding of diseases in general; but the longer we live, the more can get wrong in such a complex network. Cancer, a term used to classify a vast number of genetic and epigenetic diseases, is a consequence of deregulation of these networks, and the second leading cause of the death worldwide. According to prospective studies of the World Health Organization, one in three will develop cancer in their lifetime, while one in five will die from it (Stewart & Wild 2014).

1 CANCER

What is cancer? As beforementioned it is a blanket term for a multitude of diseases featuring uncontrolled cell growth. Cancer is practically a given outcome in every multicellular organism since every somatic cell shares the same genetic information and thus the potential to proliferate (Martincorena & Campbell 2015). A paradigm in cancer research established in the past 100 years is the somatic mutation theory of carcinogenesis, describing sporadic cancers (95% of all cancers) (Soto & Sonnenschein 2004). The quintessence of this theory states that mutations acquired over time in a **single** somatic cell give rise to a tumor, which implies, at least in the early stages of tumor progression, monoclonality. While studying these mutations two important concepts were developed: 1. Oncogenes were identified through gain-of-function studies, where mutation in the gene drive transformation into cancer (Stewart & Wild 2014). 2. Tumor-Suppressor genes were identified through the studies of hereditary cancer, where

mutations inactivate the gene function contributing to the transformation into cancer (Stewart & Wild 2014). The influential landmark review "The hallmarks of cancer" by Hanahan & Weinberg in 2000 sought to simplify and identify the essential, through mutations acquired capabilities, which unifies all cancerous diseases. The six hallmarks were 'self-sufficiency in growth signals,' 'insensitivity to anti-growth signals,' 'evading apoptosis,' 'sustained angiogenesis,' 'limitless replicative potential' and 'tissue invasion and metastasis'; notably only the last hallmark distinguishes, in general, benign from malignant growths. Cancer research accelerated in the following decade; improvements in sequencing techniques led to a revolution in cancer genomics. As a consequence, researchers were able to discriminate mutations further, labelling those, which cause a proliferative advantage and promote cancer development as "driver mutations," in contrast to "passenger mutations," which do not promote tumorigenesis (Bozic et al. 2010). The further research progressed the clearer it became that the view on cancer as a homogenous mass of proliferating cells was an oversimplification. The follow up review of Hanahan & Weinberg: "Hallmarks of cancer: the next generation" in 2011 extended, to a degree, the necessary properties of malignant transformation, by emphasizing the significance of the interaction between the tumor and normal cells, namely the role of the immune system, inflammation and altered tumor cell metabolism, especially in the tumor microenvironment (Hanahan & Weinberg 2011). Figure 1 schematically describes major characteristics that cells acquire and modifications they are subjected to for cancer to develop and progress. Nevertheless, with new whole-genome and whole-transcriptome sequencing techniques on the single-cell level and sufficient coverage to identify point mutations, the field has started to decompose tumor populations, analyze rare cell populations associated with tumor progression and study cancer evolution through lineage tracing (Baslan & Hicks 2017).

1.1 CANCER STEM CELLS & TUMOR HETEROGENEITY

This deconvolution of tumors resulted in the identification of Cancer-stem cells (CSC), a subpopulation of cells with tumor-initiating ability, which are believed to contribute to chemotherapy resistance, metastasis and disease relapse (Batlle & Clevers 2017). Insights of hematopoiesis were paving the way for the establishment of a CSC model, which in a way reflects hematopoietic stem cell development. Here are the major assumptions of this hypothesis: 1. The tumor heterogeneity is largely a consequence of the hierarchical tissue organization, which is often a reflection of the tissue of origin. 2. Self-renewing CSC, which typically account only for a small fraction of the tumor, provide the supply of cells; the bulk of the tumor consists of non-CSC cells, which have only limited proliferative capacity. 3. CSC identity is fixed; there is only limited plasticity. 4. CSC are therapy resistant and thus are a

major cause of relapse (Kreso & Dick 2014). Lately, this model was revised, since extensive lineage tracing experiments have shown evidence for a greater plasticity. This hints towards the existence of a CSC niche, in which CSC compete for niche space and also some of the differentiated cells can undergo dedifferentiation when exposed to the niche signals (Plaks et al. 2015; Batlle & Clevers 2017). Understandably, CSCs have become highly relevant targets in cancer research. It was proposed that high autophagic flux is one of the key traits of CSCs helping them to withstand harsh conditions of hypoxia and nutrient deprivation (Marcucci et al. 2017). Autophagy modulation could thus sensitize CSC to anticancer therapy. The other resistance mechanisms in CSC include, but are not limited to, an increased drug-efflux through membrane transport, a heightened capacity to withstand oxidative stress through aldehyde dehydrogenase activity and an enhanced DNA damage response (Cojoc et al. 2015).

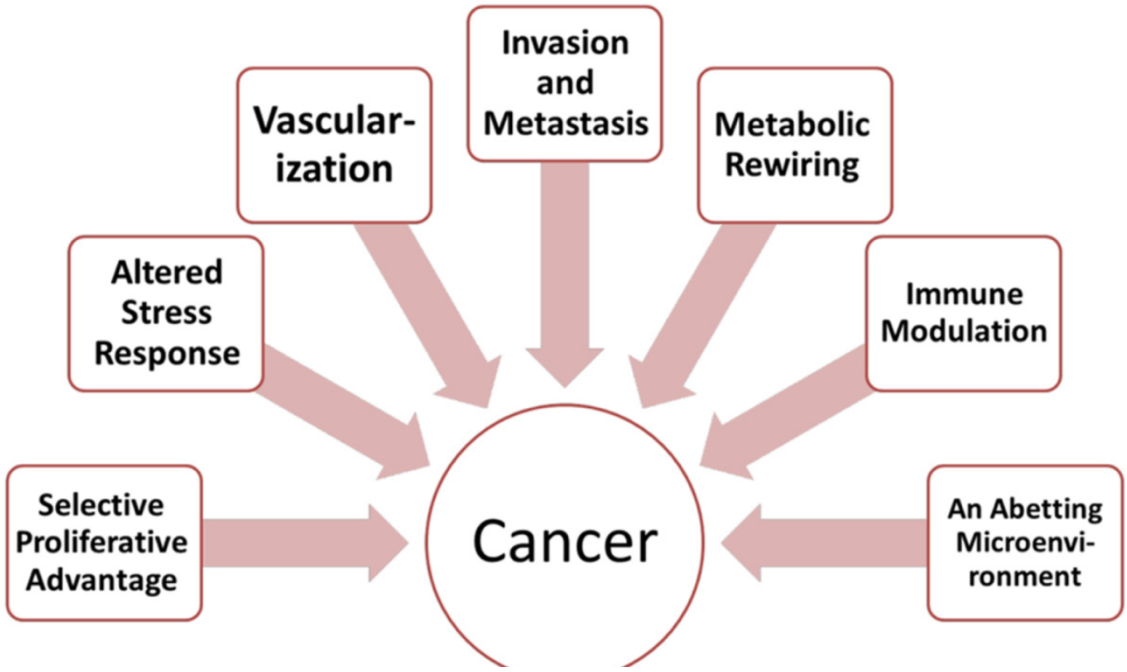

Figure 1: Major characteristics that cell acquire and modifications they are subjected to for cancer to develop and to progress.[8]

1.2 BREAST CANCER

Breast cancer is the second most common cancer worldwide and the leading cause of cancer mortality in women. Initially oncologists attempted to distinguish breast cancer subtypes solely on histopathology, which resulted in 70-80% of all breast cancer cases falling only into one of three categories, namely invasive lobular carcinoma, invasive ductal carcinoma or 'not otherwise specified' (Viale 2012). Subsequently, a biological classification for early breast cancer was established to predict the response to anticancer therapy in multiple consensus conferences in St. Gallen. Immunohistological staining of the proliferation marker Ki-67, and expression levels of estrogen- and progesterone-receptor proved to be valuable tools in

deciphering breast cancer subtypes (Gnant et al. 2011). Extensive genomic and bioinformatic analysis redefined the classification based on gene expression, resulting in an intrinsic molecular classification established through microarray profiling (Sorlie et al. 2003). This organization led to a better understanding of breast cancer biology, a better stratification of patient groups resulting in heightened therapeutic success and longer patient survival (Prat et al. 2015). The molecular subtypes are ER-positive (luminal), human epidermal growth factor receptor 2 (HER2)-enriched, basal-like and normal-like. The luminal subtypes are further distinguished in luminal A and luminal B, which differ especially regarding proliferation, with the latter being more proliferative, and also less sensitive to hormone-based treatment and more sensitive to chemotherapy than luminal A tumors (Ignatiadis & Sotiriou 2013).

Even within these sub-types, a varying degree of heterogeneity has been observed at both molecular, genomic and phenotypic level (Karthik et al. 2017) . Although this heterogeneity may not at the moment have a large impact on the molecular diagnostics, it will provide an important guidance for the formulation of personalized anti-cancer treatment and for the optimal use of targeted therapy (see below).

1.3 ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia, ALL, is the most common malignancy in children manifested by an expansion of immature B or T cells (Inaba et al. 2013). ALL is a heterogeneous disease with more than eleven genetic sub-groups that have been described for only pre-B-ALL (Downing et al. 2012). Advances in diagnostic tools, such as detection of recurring genetic abnormalities and multi-factorial FACS analysis lead to a successful combination of risk-based stratification and multi-agent chemotherapy, which has significantly increased survival of the patients in recent years. The current stratification of ALL is based on genetic abnormalities (Pui et al. 2015). Interestingly, however, they are insufficient to fully explain ALL pathogenesis as they, on their own, fail to induce leukemia in *in vivo* models (Inaba et al. 2013). This inconsistency may indicate that additional yet uncovered factors are involved in the ALL pathology. Despite the advances in the treatment, 10-15% of patients will still relapse. ALL relapse has been linked to the persistence of stem-like progenitor cells (Mullighan et al. 2008), which are less proliferating and are not readily eliminated by the treatment. These cells, first discovered in acute myeloid leukemia, AML, may represent the subset of cells that have been shown to be able to engraft in mice in patient-derived ALL xenograft (PDX) models (Meyer et al. 2011). The relapsed disease becomes highly resistant to therapy, and a high proportion of patients with relapse will die of the disease.

Despite carefully monitored chromosomal translocations and other genetic changes in ALL, treatment protocols of pediatric ALL in Sweden do not include any targeted therapy based on these genetic, but rather cytotoxic drugs that are directed against particular biological features of these leukemic cells. These features include the inability to produce L-asparagine (resulting in high sensitivity to the enzyme L-asparaginase) and the high sensitivity to metabolic hormones glucocorticoids (GC) dexamethasone or prednisolone (Pui et al. 2015). It is still unknown what are the mechanisms underlying the high sensitivity of leukemic cells to GCs. Pre-B-ALL cells were shown to rely rather on glycolysis for energy production instead of oxidative phosphorylation (OXPHOS), and they express high levels of glucose transporters on their surface (Boag et al. 2006). The switch to glucose metabolism for energy production may depend on the commonly occurring mutations in transcription factors PAX5 and IKZF1, which otherwise act as metabolic gatekeepers in this stage of B-cell differentiation (Chan et al. 2017). As GCs regulate glucose uptake and metabolism in different tissues, this may underlie their pronounced cytotoxic effects on ALL cells that highly depend on glucose metabolism. Thus, perhaps not surprisingly, GC resistance in primary ALL cells was associated with an increased expression of glucose metabolism genes and enhanced glycolysis (Hulleman et al. 2009; Holleman et al. 2009). However, the mechanisms of GC-induced cell death in ALL remains not fully understood.

1.4 CANCER THERAPY

Anti-cancer therapy was dominated by surgery and radiotherapy up until the 1960s. These methods were effective on a short-term, but remission rates plateaued at around 30% (DeVita & Chu 2008). Due to military mustard gas experiments in the middle of the 20th century (Connor 2018) and the subsequently depletion of lymph nodes and bone marrow observed in treated subjects, one of the first chemotherapeutics was discovered: nitrogen mustard derivatives that target bone marrow and leukocytes and were since successfully used in therapy of lymphomas (DeVita & Chu 2008). The development of animal models, especially the establishment of a transplantable tumor system in rodents led to the possibility to test novel compounds for anticancer properties (Grever et al. 1992). Thus initially, newly discovered drugs were cytotoxic to fast proliferating cells in general, hence as a side effect also immunosuppressive. Most of the discovered compounds were used against hematological malignancies, but the observation that rat hepatoma cells have an increased uptake of Uracil led to the first drug, which is up until today part of some treatment protocols against solid tumors: 5-fluorouracil (HEIDELBERGER et al. 1957).

Although governed by common mechanisms, each cancer disease is characterized by features specific to each individual tumor and therefore individual treatment regimen needs to be composed for each patient. This approach, called personalized cancer medicine, is being applied worldwide including Karolinska hospital (Jackson & Chester 2014). Specific mutations in such genes as tyrosine kinases led to a discovery of tyrosine kinase inhibitors that have revolutionized anti-cancer treatment. Inhibitors against BCR-ABL, EGFR, VEGFR, HER2, some of them used in this thesis, and many others have been developed (Neal & Sledge 2014). At the same time, novel therapeutic targets for cancer treatment need to be explored to match tumor variations and heterogeneity in cancer management. Moreover, cancer cells are highly adaptable, developing mutations or activating survival pathways in response to treatments (Fisher et al. 2013). One of such pathways is autophagy, the subject of this thesis. The development of novel therapies targeting autophagy is subject of one of the papers in this thesis, as well as an attempt to overcome drug resistance to tyrosine kinase inhibitors using autophagy inhibitors.

2 AUTOPHAGY

Autophagy is an evolutionary conserved catabolic process, in which cell own constitutes (damaged organelles, protein aggregates) are recycled by delivering them to lysosomes. This 'self-cannibalization' was first reported in mammalian cells more than five decades ago (Novikoff & Essner 1962). This fundamental process is observed in nearly every eukaryotic cell (Reggiori & Klionsky 2002), but it is highly tissue and context specific; hence basal levels of autophagy and the dependence on the cell origin in this process vary between different tissues (Mizushima & Komatsu 2011).

A critical function of autophagy in cellular homeostasis is protein and organelle quality control (Mizushima et al. 2008), but it nevertheless took many years to recognize its beneficial role in the cell as a recycling mechanism. The observation that dying cells often increase autophagosome generation led to a premature stigma: the association with cell death, which led to the description of autophagy as a Type II Programmed Cell death. Although parts of the autophagy machinery have been reported to be directly involved in apoptotic cell death (Denton et al. 2012; Grandér et al. 2009), the cytoprotective functions of autophagy under different stress stimuli, i.e. starvation, metabolic stress, Reactive Oxygen Species (ROS) generation or proteotoxicity, is undisputed (Mizushima & Komatsu 2011).

Until 1990's little was known about the autophagy pathway, and research was limited to phenotypical studies only. It was the work of the Ohsumi Lab, which paved the way in

understanding the proteins involved in autophagy, earning Yoshinori Ohsumi the Nobel prize in 2016. Having established a simple, yet elegant model system in yeast cells enabled him to identify many AuTophaGy-related genes (ATG) (Mizushima 2018).

Autophagy has a significant impact on metabolism and protein degradation, which can influence half of the proteome of a cell (Mathew et al. 2014). Unsurprisingly, many human pathologies are associated with abnormalities in autophagy (Figure 2). Autophagy is being divided into three major types: Macro-, Micro- and Chaperone-mediated- autophagy. Both Micro- and Chaperone-mediated autophagy utilize a direct translocation of cargo into the lysosome. The former, by direct engulfment of the cytoplasm, the latter utilizes chaperons, which recognize target proteins (Mizushima et al. 2011). This doctoral thesis solely focuses on macroautophagy.

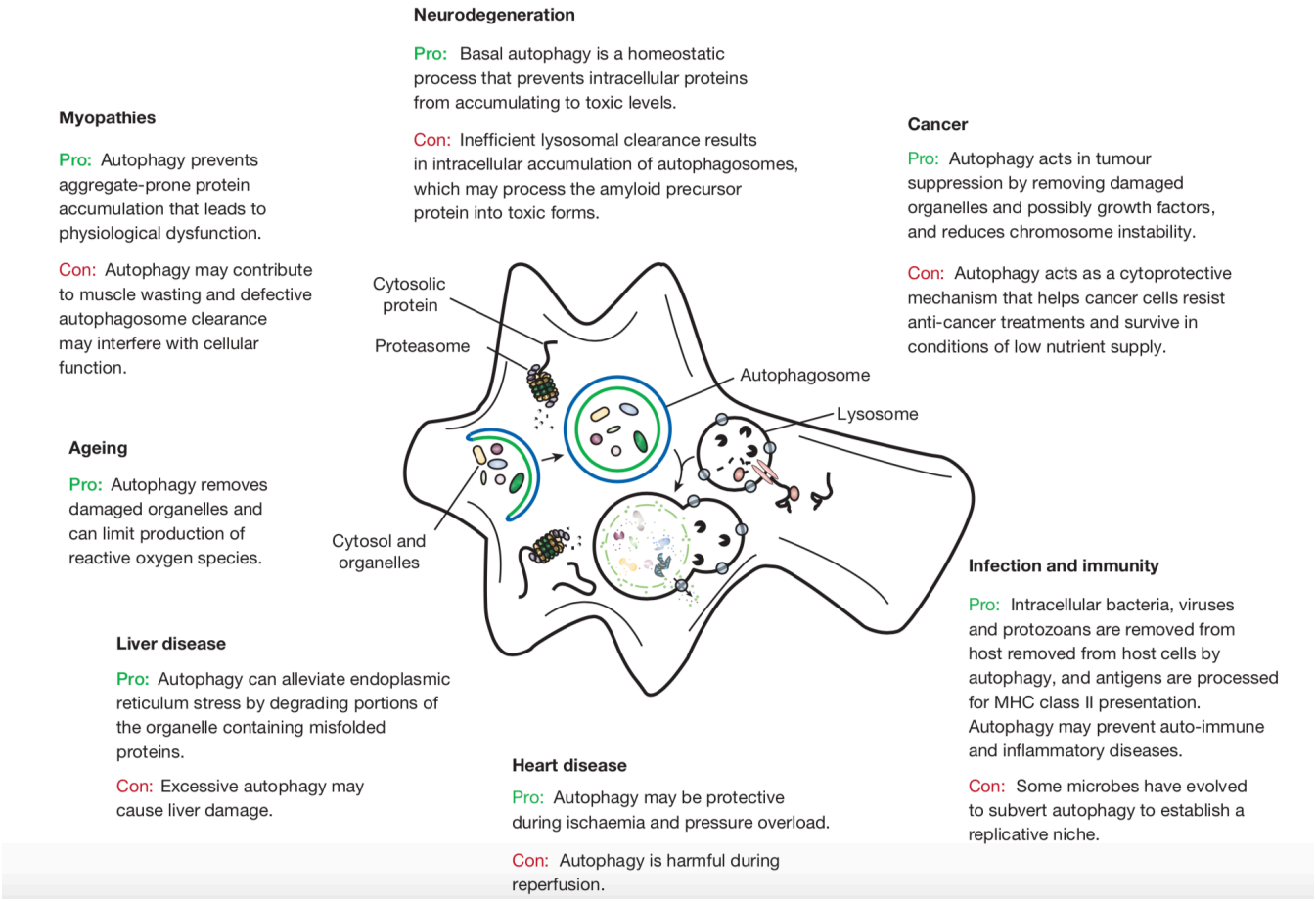

Figure 2: Role of Autophagy in different tissues *(Mizushima et al. 2008)*

Three steps characterize autophagy:

- **Initiation & Nucleation**. The formation of a double-membrane vesicle called isolation-membrane or phagophore.

- **Elongation & Closure**. The elongation of the double membrane around specific cargo or cytosol. Characterized by Microtubule-associated protein 1A/1B-light chain 3 (LC3) lipidation and its incorporation into the autophagosome membrane.
- **Maturation and degradation**. Fusion with endosomes and ultimately lysosomes, which provide the catalytic enzymes for the breakdown of the cargo.

Next, to this division, there are two observed *modi operandi*: Selective and nonselective autophagy. Non-selectiveness in this context means the formation of the autophagosome unspecifically so that the content of the autophagosome is virtually indistinguishable from the surrounding cytoplasm. In this case, bulk protein degradation helps to supply the cells with free building blocks to overcome stressful periods such as starvation. Selective autophagy, on the other hand, describes the targeted degradation of proteins or cell organelles using autophagy adapters such as ubiquitin-binding protein p62/Sequestosome-1 (p62/SQSTM1; subsequently called p62) and Neighbor of Brca1 gene (NBR1). The highly specialized autophagosomes are selectively sequestering cargo; the content is concentrated, and the cytoplasm is to a high degree absent. The autophagic adapters such as p62 determine the specificity of this process by bridging the gap between the to-be-degraded substrate and the core autophagy proteins via interaction with autophagy receptors such as LC3. The nomenclature of the different types of selective autophagy reflects the recognition and degradation of the particular cargo, i.e., Lipophagy for degradation of lipid droplets; Mitophagy for degradation of Mitochondria; Xenophagy for degradation of microbes and viruses (Rogov et al. 2014). Selective autophagy plays a critical role in the quality control of proteins and cell organelles. It is, for example, the sole mean of degradation of dysfunctional mitochondria, a source of DNA-damaging ROS and pro-apoptotic proteins (Rogov et al. 2014).

2.1 REGULATION OF AUTOPHAGY

More than 30 proteins have been identified, which play a direct role in canonical autophagy, not mentioning here any of the lysosomal proteins. However, instances of non- canonical autophagy have been reported, which can occur independently of some critical components of the core-machinery (Codogno et al. 2012).

2.1.1 Initiation – ULK1 and Beclin1-VPS34

Two multi-protein complexes are essential in the initiation of canonical autophagy. The first complex is the Unc- 51 Like Autophagy Activating Kinase 1 (ULK1) complex, which is negatively regulated by the mechanistic Target Of Rapamycin (mTOR), a central pathway

modulating protein synthesis, cell growth and cell cycle progression (Figure 3). If nutrients or growth factors are present, mTOR phosphorylates ULK1 rendering the autophagy initiation complex inactive. Under starvation conditions this inhibitory signal is absent, and the ULK1 complex can phosphorylate Beclin-1 enabling the formation of the second multi-protein complex, essential for the initiation of canonical autophagy. Beclin-1 has a Bcl-2 homology 3 (BH3) domain, which gives it an ability to interact with members of the B-cell lymphoma 2 (BCL2) family of proteins like BCL2, which is an anti-apoptotic protein and an autophagy inhibitor. Thus, under non-starved conditions, Beclin-1 is bound to BCL2 and is not able to recruit any proteins for complex formation and autophagy initiation. The phosphorylation by ULK1 disturbs this connection, and Beclin-1 can recruit Vacuolar protein sorting 34 (VPS34) and other proteins such as VPS15, Activating molecule in BECN1-regulated autophagy protein 1 (Ambra1), and ATG14L as shown in Figure 4.

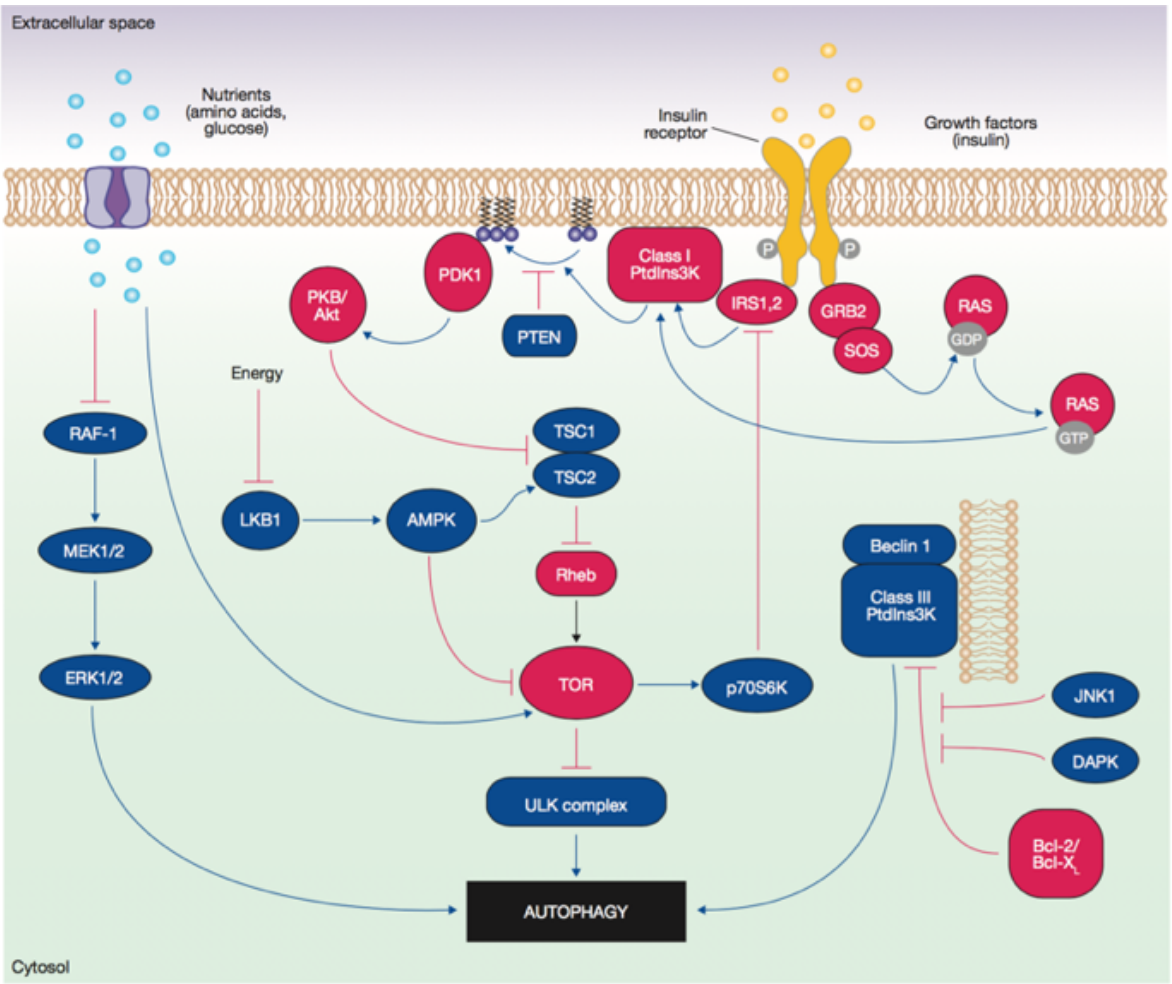

Figure 3: Cell signaling regulating Autophagy. Blue color: Autophagy promoting factor; Red color: Autophagy inhibiting factor. (Brown et al. 1994)

VPS34 is a Class-III PI3Kinase, which phosphorylates phosphatidylinositols producing phosphatidylinositol 3-phosphate [PI(3)P], a phospholipid essential for phagophore formation (Jaber et al. 2012). Members of the WD-repeat protein interacting with phosphoinositides

(WIPI) can subsequently bind the PI(3)P in the phagophore membrane and work as effectors in elongation and maturation of the autophagosome (Proikas-Cezanne et al. 2015).

Depending on the cofactors bound to the Beclin-1-VPS34 complex, its function can be modulated. Thus, the presence of Ambra1 and ATG14L play an essential role in the initiation of the phagophore. On the other hand, when instead of these co-factors UVRAG is present in the complex, a role of the complex in autophagosome maturation has been observed (Funderburk et al. 2010). A third complex, called CII (as opposed to CI involved in autophagy) has been described, involved in endosomal maturation and trafficking (Levine et al. 2015).

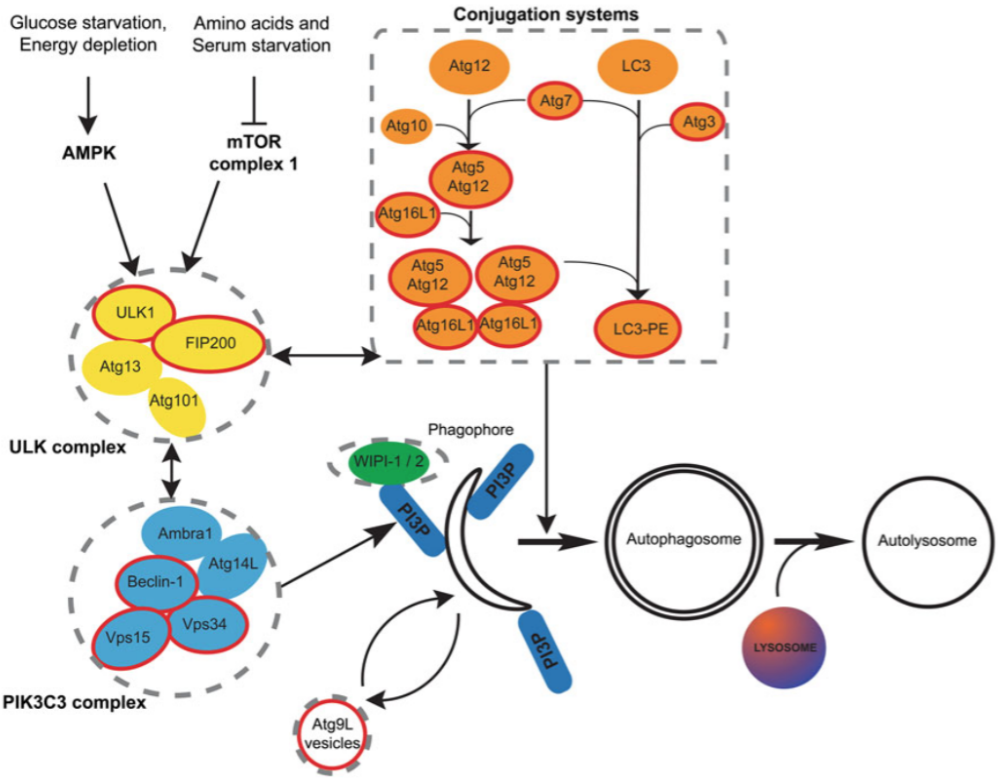

Figure 4: Canonical Autophagy Core Machinery (Dupont & Codogno 2013)

2.1.2 Elongation/closure – two Ubiquitin-like conjugation systems

It is often fascinating to observe how nature and evolution conserve specific complex and highly functional systems, which are modified and reused in a different context. An example of this can be found in autophagy manifested by the two Ubiquitin-like conjugation systems ATG12 and LC3. The resemblance to the Ubiquitin-Proteasome-System (UPS), which is used for the targeted degradation of short-lived proteins, is striking.

These two systems work in unison to achieve the lipidation of Microtubule-associated protein 1A/1B-light chain 3 (LC3), so it can be incorporated in the growing autophagosomal membrane (Figure 4). It has been suggested that LC3-lipidation is driving the expansion of the autophagosomal membrane. Different autophagy receptor and adaptor proteins, such as p62,

optineurin or NBR1 can bind to LC3 via an LIR-motif (LC3-interacting-region) and deliver cargo to the autophagosome. How does this lipidation occur? ATG7 (an E1-like Enzyme) and ATG10 (an E2-like Enzyme) covalently bind ATG5 to ATG12. ATG5–ATG12 forms subsequently a tetramer with ATG16L1, which is an E3-like Enzyme essential for the last step of the LC3 conjugation (Eisenberg-Lerner et al. 2009).

2.1.3 Maturation and degradation

After the formation of the autophagosome, multiple fusion events with endosomes and lysosomes lead to the maturation of the autophagosome, then called autolysosome. The fusion is mediated via soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes (Itakura et al. 2012). The cargo and the inner membrane of the autolysosome will be degraded utilizing the hydrolytic enzymes of the lysosome (Mizushima & Komatsu 2011). The resulting free fatty acids, sugars, amino acids and nucleosides/nucleotides can be used to fuel the cell metabolism and sustain macro molecule *de novo* synthesis (Rabinowitz & White 2010).

Autophagy plays a vital role in cellular homeostasis. It is not surprising that the impact of autophagy on cancer is manifold and double-edged as in other autophagy-related diseases: Autophagy can act as a potent tumor suppressor, but also as a tumor benefactor, sustaining tumor progression (Figure 2). Therefore, it is crucial to especially assess the role of autophagy in the disease outcome at the stage of cancer progression. The next session will describe in detail, how autophagy can either suppress tumor development or contribute to tumor progression.

2.2 MODULATION OF AUTOPHAGY

2.2.1 Nutrient starvation and mTOR

A key regulator of the transcription of genes involved in autophagy is the nutrient sensor mTOR complex 1 (mTORC1). The consensus knowledge is that upon accumulation of amino acids (AA) in the lysosomal lumen, v-ATPase interaction with RagB/D transmits an activating signal recruiting mTOR to the lysosomal surface where it gets activated by Rheb (Zoncu et al. 2011). Leucine was reported to be the most potent activator of mTOR, independent of cell type, but other AAs are still required (Meijer et al. 2015). Apart from the previously described substrates, such as ULK1/2, does the serine/threonine protein kinase mTOR phosphorylate transcription factors, such as basic helix-loop-helix leucine zipper transcription factor EB (TFEB) and FoxO under nutrient-rich conditions leading to their retention in the cytoplasm and thereby inactivation (Lapierre et al. 2015). Upon a direct mTOR inhibition by small molecules

or in response to starvation, these transcription factors localize to the nucleus and can activate gene transcription; TFEB genes' activity leads to the activation of autophagy and lysosomal biogenesis (Settembre et al. 2011).

2.2.2 Inhibition of autophagy

2.2.2.1 Late inhibitors

It is of importance to distinguish between two types of inhibition of autophagy. The autophagic flux can be blocked by late stage inhibitors. This is done by interfering with lysosomal function or by blocking the fusion of the autophagosome with the lysosome. Acid-protease inhibitors, such as Pepstatin A and E64d inhibit multiple cathepsins and effectively suppress lysosomal function (Jung et al. 2015). V-ATPase inhibitors such as BafA1 and concanamycin interfere with the proton pump and block the acidification of the lysosome (Huss & Wieczorek 2009). Lysosomotropic agents, such as chloroquine (CQ) or hydroxychloroquine (HCQ) or its derivatives, like Lys-05 also prevent the acidification of the lysosome. These agents freely diffuse through the plasma membrane of the lysosome, get protonated by the H+ and get trapped, which leads to an increase in pH and a lysosomal dysfunction (Pasquier 2015). All these drugs lead to an accumulation of autophagosomes and dysfunctional lysosomes, a state, which has been shown to confer cell cytotoxicity (Button et al. 2017).

2.2.2.2 Early inhibitors

Early autophagy inhibitors target proteins involved in the early stage of the autophagy process. The Pan-PI3K inhibitor 3-Methyladenine was the first discovered compound, which inhibits VPS34, a Class III PI3K, and therefore the initiation and maturation step of autophagy. Since it is a pan-PI3K inhibitor also affecting PI3K class I, which has the opposite to VPS34 effect on autophagy, it would simultaneously induce autophagy, leading to some unclear effects (Y.-T. Wu et al. 2010). This limits the usefulness of the pan-PI3K inhibitors regarding inhibition of autophagy. Other noteworthy pan-PI3K inhibitors are Wortmannin and LY294002 widely used in research. More specific inhibitors of VPS34 have been developed to overcome these issues. Catalytic inhibitors of VPS34 were developed by Sanofi and Novartis; while Sanofi discovered a pyrimidinone, with the name SAR405, selective for VPS34, Novartis found a bisaminopyrimidine, named PIK-III, also with a high selectivity profile against VPS34 (Pasquier 2015). I describe in this thesis the activity of SB02024, a new VPS34 inhibitor developed by Sprint Bioscience. SB02024 is a potent compound that selectively targets VPS34, has good pharmacodynamics properties and significantly reduces the growth of human tumor xenograft in mouse models (Paper III).

ULK1/2 has been shown to be another potential target to enable an early pharmacological autophagy inhibition. It has been shown that ULK2 can substitute ULK1 functions in certain cell types (Lee & Tournier 2011), which increase the necessity for a dual ULK1/2 inhibitor. MRT67307 is a compound discovered by the Ganley group, which seem to have these properties, also including a high potency (Petherick et al. 2015).

3 ROLE OF AUTOPHAGY IN CANCER

Cancer is a complex disease, where an accumulation of mutations or epigenetic changes underlie cancer development and progression. Oncogenes, which drive proliferation; dysfunctional tumor-suppressors, which fail to constrain the transformation of the cell; the immune system, which fails to recognize the abnormality: these are many factors necessary for the tumor progression. In recent years, a growing understanding of the profound changes in cancer cell metabolism led to a new perspective: to view cancer as a metabolic disease. On all these levels autophagy has an impact summarized in Table 2. It is undisputed that this impact can have positive and negative consequences for the pathogenesis of cancer. It lies in the hand of researchers to understand the interplay between autophagy and cancer and find the right target in the right context to exploit this knowledge for therapeutic purposes. The next section will focus on the dual role of autophagy in cancer.

3.1 AUTOPHAGY AS A TUMOR SUPPRESSOR MECHANISM

Extensive *in vivo* experiments utilizing genetic inhibition of autophagy core genes in various tissues demonstrated the role of autophagy as a tumor suppressor in cancer initiation as the knock-outs of many of the genes involved in regulation of autophagy lead to tumorigenesis in a variety of tumor models (Table 1).

3.1.1 Genomic stability

There are several ways of how autophagy contributes to the genomic stability. For example, Mitophagy can remove dysfunctional mitochondria, which is a source of ROS, thus reducing genotoxicity (Takahashi et al. 2013). Whenever chromosomes or fragments of chromosomes are not correctly incorporated in the daughter nuclei, small bodies of the nucleus are formed called micronuclei. Autophagy is a standard way for cells to dispose of micronuclei, thereby contributing to genomic stability (Rello-Varona et al. 2012). Retrotransposons, genetic elements, which can incorporate themselves in the genome using a “copy and paste” mechanism, are potentially disrupting genetic stability. This integration occurs with the help of an RNA intermediate, which is susceptible to autophagic degradation; hence autophagy contributes to genomic stability (Guo et al. 2014).

Table 1: Murine models with various knock-outs of key components of the autophagy signaling and their phenotype.

Gene target	Phenotype	Ref
AMBRA1	Spontaneous tumorgenesis	Cianfanelli et al, 2015
ATG4c	Fibrosarcoma	Marino et al, 2007
ATG5 (mosaic)	Benign hepatic neoplasms	Takamura et al, 2011
ATG5/7 (lung specific)	KRASG12D lungcarcinoma	Strohecker et al, 2013
ATG5/7 (lung specific)	BRAFV600E lungcarcinoma	Rao et al, 2014
ATG5/7 (pancreas specific)	KRASG12D pre-malignant pancreatic lesions	Rosenfeldt et al, 2013; Yang et al, 2014
ATG7 (liver specific)	Benign hepatic neoplasms	Takamura et al, 2011
ATG7 (hematopoietic stem cells)	Neoplastic bone marrow	Mortensen et al, 2011
BECN1+/-	Lymphoma, lung & liver carcinoma, Wnt1-driven carciogenesis	Liang et al, 1999; Qu et al 2003; Yue et al, 2003; Mortensen et al, 2011,(22)

3.1.2 Oncogenes / Tumor-suppressors

Multiple reports imply that autophagy receptor p62 has an oncogenic function. It is usually degraded via the autophagic pathway; its ectopic overexpression (or its presence due to autophagy impairment) can stabilize oncoproteins such as Twist1 (Qiang et al. 2014) or act as a signal transducer for NF-**κ**B signaling (Duran et al. 2008). p62 has also been shown to be responsible for metabolic changes in hepatocellular carcinoma. In a recent study it was shown that phosphorylated p62 could activate the transcription factor Nrf2, which directs glucose to the glucuronate pathway and glutamine towards glutathione synthesis; both increasing chemotherapeutic resistance and proliferative capacity of the cancer cell (Saito et al. 2016). Thus, keeping p62 levels low via active autophagy may contribute to tumor suppression.

Activation of an oncogene is connected to tremendous stress for the cell. This strain is usually countered by oncogene-induced cell death (OID) or oncogene-induced senescence (OIS) in normal cells. It has been shown that deletion, knockdown or inhibition by small molecules of several components of the autophagy core machinery (mainly targeting Beclin-1, ATG5 or ATG7) can limit both OID and OIS (Young et al. 2009), again suggesting a function of autophagy in tumor suppression.

A report investigating the correlation between low Beclin-1 expression in WNT1-driven breast cancer and aggressiveness implied a function for Beclin-1 in a non-autophagic role in mammary development (Cicchini et al. 2014).

Reports that autophagy is taking part in the degradation of the mutant, but not the wild-type TP53 suggests another tumor suppressive function of autophagy. Mutant TP53 can exert a dominant-negative effect by blocking the function of the remaining wild-type TP53 allele in heterozygous cells. It was shown that ectopic expression of Beclin-1 or ATG5 lead to a reduction of mutant TP53 levels, while knock-down of these autophagy genes via RNA-interference (RNAi) lead to accumulation of mutant TP53 (Choudhury et al. 2013).

3.1.3 Immune response

One first line of defense of the innate immune system are macrophages residing in tissues and continuously scanning their environment; as soon as signals are received, they would trigger an immune response. Tissue damage, for instance, can lead to the release of danger-associated molecular patterns which would activate such an acute inflammatory response; pathogens, invading the tissue, can be recognized by the macrophages due to pathogen-associated molecular patterns. The macrophages get also activated to dispose of dead cells and initiate tissue repair and regeneration. This activity is accompanied by mitochondrial stress, due to ROS production and proinflammatory cytokine release. Overactivation of macrophages can lead to prolonged inflammation and thereby can contribute to oncogenesis. Autophagy is one way to counteract this process, by degrading mitochondria and thereby reducing ROS production.

Immune evasion by the tumors is one the hallmarks of cancer; it is necessary for the malignant tumors to stay undetected by the antigen-presenting-cells (APCs), which otherwise would promote their destruction via innate and adaptive immune systems. It has been shown that autophagy-mediated release of ATP can recruit and activate APCs, thus increasing tumor antigen recognition by these cells. Indeed, a decreased autophagy through a decreased ATP secretion could prevent tumor cell detection by the immune system (Wang et al. 2013).

3.2 AUTOPHAGY AS TUMOR BENEFACTOR

Although autophagy plays a suppressive role in early tumorigenesis (see above) it turned out to be necessary for established tumors to survive various cellular stresses such as lack of nutrients, hypoxia, low pH and Endoplasmatic Reticulum (ER) stress (Figure 5). The prerequisite for metastasis and invasion, two features of tumor progression, is the loss of cancer cell adhesion, providing the ability to migrate manifested by epithelial-mesenchymal transition (EMT). Autophagy that was shown to be able to prevent anoikis (Kenific & Debnath 2015), can thus contribute to EMT. Indeed, inhibition of autophagy was shown to limit metastasis and

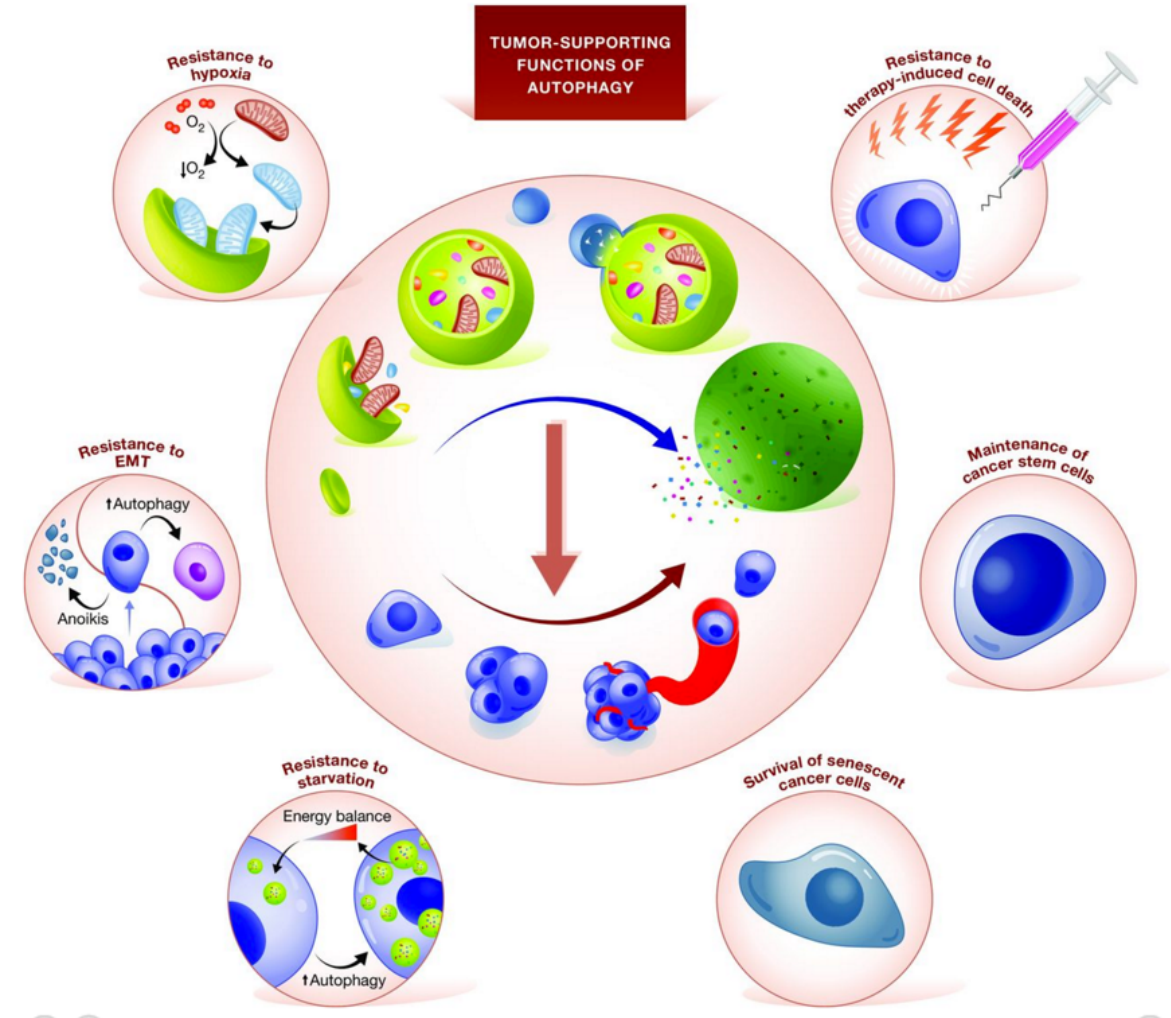

Figure 5: Autophagy in malignant transformation *(Galluzzi et al. 2015)*

invasiveness and therefore the progression of cancer to more advanced stages (Galluzzi et al. 2015). High levels of autophagy in tumors were shown to correlate with a poor disease outcome (Lazova et al. 2012) strengthening the role played by autophagy in the maintenance of tumor cell survival. Autophagy is also vital for the maintenance of cancer stem cells (CSCs), which can exhibit high levels of basal autophagy. It has been shown that the tumor-forming ability of mammary CSCs is impaired in vivo when BECN1 or ATG4A is knocked down using RNAi (Wolf et al. 2013; Gong et al. 2013). A multitude of factors, which are primarily connected to

the ability of autophagy to counter cellular stress could be accounted for tumor progression (Table 2). Metabolic stress, the interaction with the tumor microenvironment and the ability to resist therapy-induced cell death are the factors this thesis will pay particular attention to.

3.3 AUTOPHAGY IN ANTI-CANCER THERAPY

3.3.1 Autophagy in radiotherapy

Two standard first-line treatments for a multitude of cancer types are chemo- and radiotherapy, aside from surgical removal of a tumor. Radiotherapy is strongly associated with autophagy. The question whether autophagy may be beneficial for the tumor cells to survive this treatment or not has been extensively discussed, as reviewed by Yang et al. Multiple reports have shown a positive correlation between upregulation of autophagy and resistance to radiotherapy. Contradictory to these reports were observations that autophagy would enhance the anticancer effect of radiotherapy and that the inhibition of autophagy would promote cell survival (Yang et al. 2015). The take-home message from these studies, each focused on a particular cell line or the corresponding tissue, is the importance of the context specificity in order to determine the role of autophagy in the treatment outcome. Even more extensive are the literature reports about the cytoprotective and cytotoxic role of autophagy under chemotherapeutical treatment, which will be discussed in the following chapter.

3.3.2 Autophagy and Chemotherapeutics

It is, perhaps, not surprising that many of the chemotherapeutic drugs that induce DNA or organelle damage, induce autophagy. Many reports show that autophagy induced by chemotherapy may protect tumor cells from cytotoxicity (Nagelkerke et al. 2015). However, some reports contradict this notion and instead suggest the importance of autophagy for therapy-induced cell death (Gozuacik & Kimchi 2007; Grandér & Panaretakis 2010). David Gewirtz tries to tackle this problem by the introduction of a new classification of autophagy in anti-cancer treatment, suggesting autophagy induced by chemo- or radiotherapy being either cytoprotective, nonprotective, cytostatic or cytotoxic (Gewirtz 2014). This categorization imposes a new level of complexity to the inherently difficult task of understanding the contribution of autophagy to therapy-resistance. It also implies the necessity to improve scientific methods, which are currently represented by the apparent use of genetic or pharmacologic inhibition of autophagy in the quest of establishing its relevance.

3.4 AUTOPHAGY AND CELL METABOLISM

3.4.1 Autophagy and metabolic stress

One of the hallmarks of cancer, so-called "Warburg-effect," implies a profound switch of energy metabolism in the cancer cell from the OXPHOS to the energetically less favorable anaerobic glycolysis. This radical reprogramming is driven by oncogenes like RAS, AKT, and MYC and causes an enormous amount of cellular stress. When OXPHOS, as well as the process of Beta-oxidation of lipids are reduced, the cell in return increases the expression of genes involved in glucose uptake and glycolysis, as well as start utilizing other sources of energy such as glutamine (Mazurek & Shoshan 2015), as depicted in *Figure 6* (The figure refers to Ahmai et al, chapter 3 in the book).

It has been postulated that autophagy in this context serves two purposes. First, it dampens the effect of the metabolic reprogramming by providing the cell with amino and fatty acids. Secondly, autophagy may provide energy sources from the tumor microenvironment. An increased glutaminolysis would lead to an increase in ammonia ions as a result of the enzymatic reactions of glutaminase and glutamate dehydrogenase. Ammonia would in turn increase autophagy in ULK1 dependent (Li et al. 2016) and independent manner (Cheong et al. 2011) . Since it is a volatile compound, it is released into the stroma thereby elevating autophagic flux in cancer-associated fibroblasts, CAFs, which in turn decreases their mitochondria numbers and thus the OXPHOS potential of these stromal cells. CAFs would have in turn to switch to glycolysis to compensate for the energy deficit, which could lead to the secretion of lactate and other high-energetic compounds that tumor cells would use as an additional energy source (Mazurek & Shoshan 2015). In concert with this model, it was reported that RAS-driven tumors show an addiction to autophagy even when external stress stimuli such as nutrient-deprivation are lacking (Maycotte et al. 2014).

It is important to mention here another aspect of autophagy in supporting the resistance of cancer cells to the stringent metabolic environment. Uncontrolled cell proliferation and unbalanced angiogenic signaling in a tumor lead to heterogeneous vascularization and therefore a limited blood supply. As a consequence, some tumor regions become continuously low on nutrient and oxygen availability leading to starvation and hypoxia, respectively. Both states are potent triggers of autophagic flux, which helps tumor cells to withstand this harsh environment. Thus, pharmacological inhibition of autophagy could lead to sensitization of human cancer cells to hypoxia (Rouschop et al. 2010).

3.4.2 Autophagy and tumor acidosis

Switch to glycolysis for energy production has its consequences. Thus, when glucose is metabolized, either aerobically or anaerobically, but the entry of pyruvate to the citric cycle is limited, it leads to the production of lactate. Moreover, ATP hydrolysis and decarboxylation reactions contribute to produce acid equivalents and protons(Corbet & Feron 2017). Cancer cells upregulate membrane transporters to effectively excrete these metabolites to maintain a physiological intracellular pH (pHi); this, however, consequently lowers the extracellular pH (pHe) in the tumor microenvironment (Gillies et al. 2008). Thus, the metabolic shift to glycolysis leads to a decrease in the pH of the tumor microenvironment. The low pHe can lead to a reduced leucine uptake, an inhibition of mTOR, and subsequently activation of autophagy (Marino et al. 2012). Multiple reports have shown that exposure to an acidic environment is a prominent trigger of autophagy induction (S. Wu et al. 2013; Marino et al. 2012; Wojtkowiak et al. 2012).

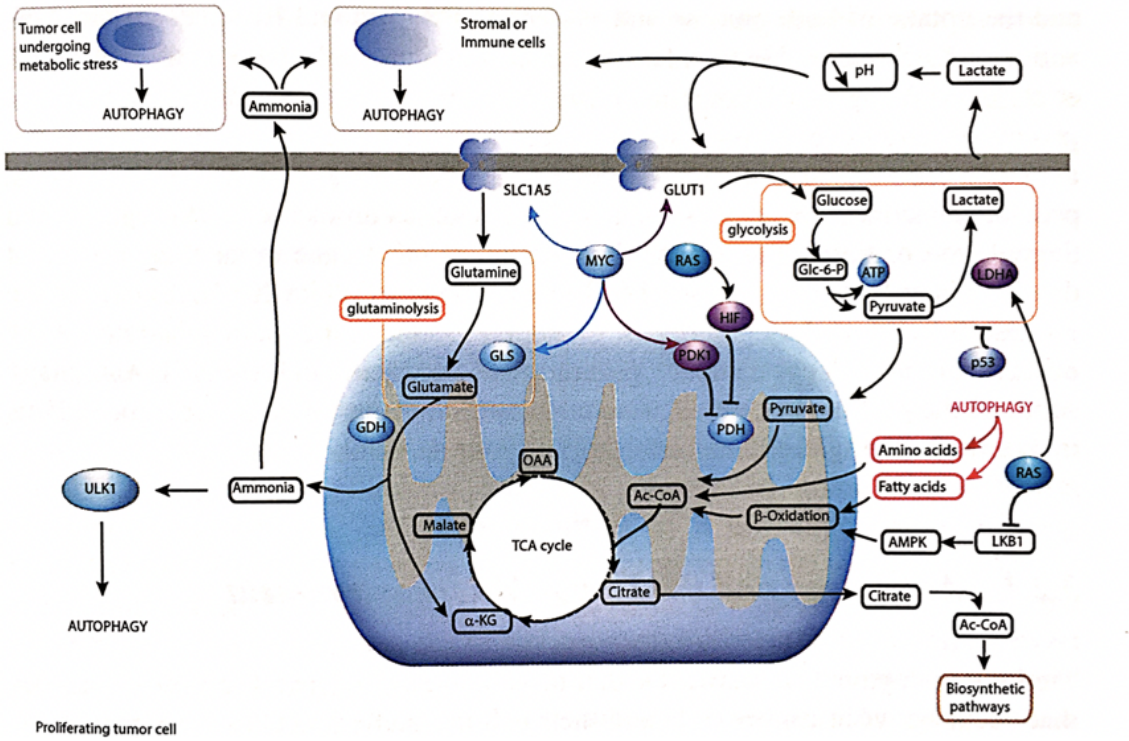

Figure 6: Altered cell metabolism in cancer (Mazurek & Shoshan 2015)

Autophagy, in turn, has an essential function in the adaption of cancer cells to acidosis, since inhibition of autophagy increases cytotoxicity in cells exposed to low pH (Wojtkowiak et al. 2012; Marino et al. 2012; Zhang et al. 2017). The precise mechanism of how autophagy helps cells to survive acidic environment is not known. It has been postulated that the involvement of autophagy in the maintenance of mitochondrial integrity could be one of the mechanisms

behind it (Namba et al. 2014). A second hypothesis is that autophagy helps the cells to cannibalize neighboring cells exposed to acidic stress, similarly to the known activity of autophagy in digesting some intracellular pathogens (Marino et al. 2012).

3.4.3 Autophagy and glucocorticoids

GCs are catabolic steroids that oppose the action of insulin in the body, and regulate energy metabolism in a variety of tissues in response to hypoglycemia, anoxia and stresses such as tissue damage (Rose & Herzig 2013). Distinct cell types respond differently to GCs: in muscle, GCs suppress glucose uptake and glycogen synthesis and cause breakdown of cell protein; in the liver, GCs induce gluconeogenesis, lipogenesis and represses fatty acid oxidation. GCs can also regulate cell differentiation, for example, in the early development of lung, in T-cell differentiation and during bone development. They can also suppress pro-inflammatory signaling and inhibit immunological responses (Coutinho & Chapman 2011). GCs are readily used in anti-cancer treatment, particularly for the treatment of blood cancers. Notably, as described in part 2.3 of this thesis, high doses of GCs are used for the treatment of leukemia. It was found in our lab and by others that GCs induce autophagy in leukemic cells (Laane et al. 2009; Swerdlow et al. 2008), indicating a catabolic state similar to nutrient starvation. Indeed, probably not surprisingly, GCs inhibit glucose uptake and metabolism also in ALL cells, and this may lead to autophagy induction (Buentke et al. 2011). However, the mechanisms of autophagy induction, as well as the role of autophagy in GC-induced apoptosis of ALL cells, is not fully understood, and one of the studies in this thesis addresses this question.

4 RESULTS & DISCUSSION

The general aim of this thesis was to investigate the role of autophagy in anti-cancer therapy, in particular in chemotherapeutics. The dual nature of autophagy in cancer, as described in the Introduction, its tissue-and context-specificity and absence of simple methods of detection in vivo obscured its role in anti-cancer treatment and hampered the development of drugs targeting autophagy. In the studies of this thesis, we focused on the induction of autophagy by anti-cancer drugs and on the alternative ways of autophagy regulation and inhibition using both known approaches and novel compounds targeting autophagy.

4.1 PAPER I

Tumor acidosis enhances cytotoxic effects and autophagy inhibition by Salinomycin on cancer cell lines and cancer stem cells.

Motivation

One consequence of the metabolic adaptation to rapid growth of cancerous cells is tumor acidosis. Autophagy has been shown to aid tumor cells in surviving the acidic environment and, also, to contribute to chemotherapy resistance. Especially a subpopulation of tumor cells, designated as cancer stem cells (CSC) have been associated with the ability to metastasize, with disease relapse and with enhanced resistance to anti-cancer therapy. These cells were shown to depend on autophagy for their survival. The only presently available autophagy inhibitors for the clinical use are lysosomal inhibitors. One such inhibitor widely used in clinical trials is Hydroxychloroquine (HCQ); however, HCQ was shown to have compromised cell uptake properties in acidic environment; thus, the search for new and useful autophagy inhibitors that target cells also in acidic conditions is imperative. Another drug that was reported to inhibit autophagy is Salinomycin (SAL); however, some contradictory reports were demonstrating both the induction as well as inhibition of autophagic flux, as its mechanism of action. This cytotoxic compound is an acidic ionophore, and therefore we sought out to investigate the cytotoxic effect of SAL treatment on several cancer cell lines in the low pH conditions as compared to normal cell culture conditions. SAL has been reported to selectively target breast CSC, which prompted us to elucidate its effect on the autophagic flux in cancer cells with an emphasis on CSCs.

Main findings

We found that SAL is a potent autophagy inhibitor, particularly in acidic conditions, in a melanoma, an osteosarcoma, and a colorectal cancer cell line. In the latter cell line, we could show that SAL, in contrast to CQ, can penetrate the acidic core of multicellular spheroids

efficiently blocking autophagic flux. The compound was also able to decrease cell viability and diminish clonogenic survival of these cells.

Further, we found that SAL efficiently blocks autophagic flux in breast cancer cells as well. CSC derived from either breast cancer cell lines, or primary tumor cells showed reduced viability and limited ability to form mammospheres under treatment with SAL, in particular, when cultured under low pH conditions. Using mass spectrometry, we could confirm that intracellular SAL accumulation is pH dependent, which mostly explained its enhanced activity under low pH conditions. Thus, SAL is a good candidate for the development of a drug for the clinical use that will inhibit autophagy and specifically target CSCs particularly in the acidic conditions of the tumor microenvironment.

Discussion

Tumor acidosis is an important but frequently overlooked feature, which has to be considered in drug development. An acidic environment fostered intracellular accumulation of SAL, and therefore this compound is a good candidate for further drug development against cancer. We, however, have not addressed the question whether the accumulation of SAL also leads to an increase in its activity and therefore it remains to be investigated. Autophagy that contributes to the resistance to chemotherapy and tumor progression is a good target for therapy. SAL is efficiently inhibiting autophagy as we showed in several cancer cell lines. There have been some conflicting reports of SAL's effect on autophagy: it might not be as contradictory as it seems, however, but merely a result of misinterpretation of the experimental results, namely of the accumulation of LC3-II, as a marker of autophagy. Unfortunately, no other markers, such as the degradation of an autophagic adaptor p62, as a measure for autophagic flux, has been used in the studies describing SAL as an inducer of autophagy (Jangamreddy et al. 2013). We also found SAL to efficiently inhibit autophagy in CSC, which are notoriously difficult to eradicate by treatment. Thus, inhibition of autophagy by SAL may underlie its cytotoxic effect on CSCs. Other reports pointed at the SAL-mediated effect on OXPHOS by inner mitochondrial membrane hyperpolarization and mitochondrial matrix acidification (Managò et al. 2015); this, however, could not entirely explain the sensitivity of CSC to SAL, despite a reported dependence of CSCs on OXPHOS (Batlle & Clevers 2017). Another, more plausible explanation could be an ability of SAL to sequester iron in lysosomes, catalyzing a Fenton reaction. This produces ROS, degrades the lysosomal membrane and leads to ferroptosis (Mai et al. 2017). Iron is crucial for CSC maintenance, and iron depletion has been used as a strategy to target CSCs in other studies (Ninomiya et al. 2017). In conclusion, our data shed new light on the mechanism of action of SAL as a blocker of autophagic flux, its CSC targeting properties

under low pH conditions and thus strengthens a prospect for its further developing into anticancer therapy.

4.2 PAPER II

Metabolic reprogramming of acute lymphoblastic leukaemia cells in response to glucocorticoid treatment.

Motivation

Glucocorticoids (GC) are a major part of the standard treatment of acute lymphoblastic leukemia (Pui et al. 2015). Despite the 50-year history of the use of GCs in the clinics, the precise mechanism of this metabolic drug's action is still unclear. Although it has been shown that GCs induced apoptotic cell death is dependent on GC-receptor mediated induction of its target genes (Holleman et al. 2009; Hulleman et al. 2009; Jing et al. 2014), it remains unknown what metabolic pathways are affected and involved in the sensitivity of ALL cells to GCs. Two previous studies in our group pointed at the inhibition of glucose uptake and metabolism and the induction of profound autophagy prior to apoptosis by the GC dexamethasone (Dex) in ALL cells that undergo apoptosis in response to this drug (Laane et al. 2009). However, not all of the responses can be explained by the inhibition of glucose uptake, and the mechanisms of the induction of autophagy and its role in the cytotoxic effects of GCs is still unclear. Therefore, in this study, we assessed the global response to Dex in kinetics in the GC-sensitive pre-B-ALL cell line by integrating metabolomics and high coverage proteomics data to elucidate the metabolic changes caused by GC treatment.

Main findings

Using integration of metabolomics and proteomics, we could confirm that GC treatment induces downregulation of cellular pathways necessary for proliferation, such as pyrimidine, purine and polyamine synthesis. Also, the metabolites derived from glycolysis in concert with the enzymes of the glycolytic pathway and the TCA cycle metabolites were inhibited. Many of these changes could be contributed to a decrease of protein levels of a master regulator of cell proliferation and metabolism, c-Myc. We also found a down-regulation of the enzymes in the fatty acid synthesis and upregulation of enzymes involved in β-oxidation. We found a robust increase of CDP-choline after 24 h treatment with Dex indicating de novo phospholipid synthesis. Further, we could detect a decrease in glutamine uptake, whereas glutamine synthesis was surging. This regulation was revealed by an increase in overall intracellular glutamine and by the increase of glutamate-ammonia-ligase (GLUL) mRNA and protein level, an enzyme that converts glutamate to glutamine utilizing ammonia ion. In this study, we also

monitored the induction of autophagy and of apoptotic cell death followed GC treatment of ALL cells. We found that by accelerating the GLUL-catalyzed reaction by either depleting culture medium of glutamine or by adding of a precursor, α-ketoglutarate reduced autophagic flux and, to some extent, reduced cell death in ALL cell lines. It was shown before that low GLUL expression was one of the three markers predictive of poor disease outcome and the relapse in pediatric ALL patients. Analysis of available data sets of GC-treated primary ALL cells grown as mouse xenografts (patient-derived xenografts, PDX) revealed that induction of GLUL by GC Dex was significant in the GC sensitive PDX and not in the resistant ones (Jing et al. 2014). Our data revealed activation of glutamine synthesis by GCs in the cells undergoing autophagy and apoptosis and suggested that induction of GLUL by GC treatment may be predictive of sensitivity to these drugs.

Discussion

This was the first study to our knowledge when the GC-induced changes in leukemic cells were investigated in combined metabolomic and proteomic-based approaches. Our data could confirm previous reports of GC-induced effects on leukemic cell metabolism, down-regulation of c-Myc, inhibition of glycolysis and TCA cycle, and the stall of cell proliferation (Buentke et al. 2011; Boag et al. 2006; Rose & Herzig 2013). This evidence validates the quality of our dataset. Some of our findings, such as an upregulation of the enzymes involved in OXPHOS pathway after prolonged exposure to GC could indicate an attempt of tumor cells to dynamically compensate for the loss of energy production through the glycolytic pathway, which is a central pathway for energy production in ALL cells (so-called Warburg effect). Similarly, induction of enzymes of the β-oxidation pathway may also reflect a metabolic reprogramming and a shift from lipid anabolism to catabolism. Also, despite this, we found that ALL cells increased synthesis of CDP choline, required for de novo synthesis of most phospholipids. This production could be due to the increased demand for intracellular membranes because of the membrane blebbing during apoptosis, but also due to an increase in autophagy, which is characterized by the formation of vacuoles surrounded by a double membrane (Girardi et al. 2011). We have studied in more detail another puzzling observation, namely a robust upregulation of glutamine synthesis, an energy-costly process, which is being induced despite an abundance of glutamine in the culture medium and the precarious situation of imminent cell death. One possible explanation could be the role of this process in ammonia regulation; the reaction, which is being catalyzed by GLUL sequesters ammonia, which can be toxic to cells (Suárez et al. 2002). It has been shown that ammonia can induce autophagy but also can block lysosomal function at higher concentrations. In any case, increase in ammonia

levels will be manifested by the accumulation of autophagic vacuoles (Eng et al. 2010). Our data indeed point at a possible role of ammonia in the induction of autophagy by GCs in ALL cells since fostering the reaction that sequesters ammonia resulted in a decrease of autophagic flux and even of cell death. These data allowed us to speculate that the induction of GLUL and the glutamine synthesis may reflect the need to sequester cytotoxic ammonia. Significant induction of GLUL mRNA by Dex in the sensitive ALL PDX as opposed to the resistant ones together with the previously suggested role of GLUL as a predictive marker allows us to propose that induction of GLUL and glutamine synthesis are involved in GC-induced cell death.

4.3 PAPER III

Autophagy inhibition by small molecule inhibitors of Vps34 improves sensitivity of breast cancer cells to Sunitinib.

Motivation

A variety of anticancer drugs, including cytotoxic chemotherapy and novel targeted agents were shown to induce autophagy (Janku et al. 2011). Multiple reports have addressed the role of this autophagy induction and shown that it protects from cell death since autophagy inhibition could increase sensitivity of different types of cancer cell lines to chemotherapeutics (Selvakumaran et al. 2013; Qadir et al. 2008; Pan et al. 2014). These findings led to the initiation of a multitude of clinical trials using hydroxychloroquine (HCQ, an FDA approved anti-malaria drug) in combination with chemotherapeutics (Onorati et al. 2018). HCQ inhibits autophagy at the late stage since it is a lysosomal blocker. These trials have shown, however, modest effects. One reason could be a poor cellular uptake of CQ in the acidic tumor microenvironment (Pellegrini et al. 2014). In addition, the autophagy-unrelated effects of CQ are known, casting doubts on its specificity regarding autophagy inhibition (Piao et al. 2017).

One aim in this study was to screen a library of all existing anticancer drugs for their potential to induce autophagy, and then to identify the drugs, which in combination with autophagy inhibition would increase their cytotoxic effects.

Secondly, we intended to develop a highly potent and specific autophagy inhibitor, to investigate the potential of selective inhibition of autophagy as compared to the lysosomal inhibition in the combinational anticancer therapy. For this, we took advantage of our screening results and used the drugs identified in the screen for the combination studies.

Main findings

We have found that 104 out of 306 anticancer drugs induce autophagy in the screening model system based on the GFP-LC3 puncta formation, a method widely used for monitoring autophagy. Of these, 16 showed enhanced cytotoxicity when autophagy was genetically inhibited using RNAi. We narrowed down the selection of the drugs to 6, which could also induce autophagy in a second similar model system, a breast cancer cell line expressing GFP-LC3. Finally, we have selected two tyrosine kinase inhibitors, Erlotinib and Sunitinib based on their performance in the screening and on the promising potential of these drugs in the treatment of breast cancer shown in the pre-clinical studies. We describe here the characteristics of a novel and potent small molecule inhibitor with selective properties against VPS34, SB02024. SB02024 inhibited breast cancer xenografts *in vivo* and increased the sensitivity of breast cancer cell lines to Erlotinib and Sunitinib *in vitro*.

Discussion

Researchers have previously attempted to identify bioactive compounds that induce autophagy, such as the screen of the Institute of Chemistry and Cell Biology (ICCB) library of and of the National Cancer Institute (NCI) mechanistic set library (S. Shen et al. 2011). In that study, a large proportion of the various compounds, including DNA damaging drugs, induced autophagy, which demonstrated the proof-of-principle. On the other hand, our screen of the FIMM drug library, containing most of anti-cancer drugs, provides data better applicable for translational research since these are the drugs actually used in clinical and preclinical practice and at physiologically relevant concentrations (Saeed et al. 2017). Further, in order to identify drugs whose efficacy was increased by autophagy inhibition, we applied siRNA-mediated knock-down of autophagy genes, ATG7 and VPS34. As a result, while 1/3 of the drugs from the library were able to induce autophagy, only 16 drugs were found to have increased cytotoxicity upon autophagy inhibition could be beneficial for the treatment outcome. We believe that the results are underestimated, most likely due to the transient nature of the RNAi approach and the dynamic nature of autophagy process. Nevertheless, based on the fact that many of the hits in the screening represented targeted therapeutic molecules, we concentrated on the two tyrosine kinase inhibitors that may have a promising therapeutic potential in breast cancer treatment. Further, we have assessed their cytotoxic effects in breast cancer cell lines in combination with pharmacological autophagy inhibition. For this, we used a selective small molecule inhibitor of VPS34, SB02024, that was developed by Sprint Bioscience. The activity of this novel compound in the xenograft models of breast cancer demonstrated that inhibition of VPS34 can be a valid strategy in breast cancer treatment. Also, combining SB02024 with Sunitinib increased Sunitinib cytotoxic effects in different *in vitro* experimental settings.

Moreover, SB02024 was able to sensitize MCF-7 and MDA-MB-231 breast cancer cell lines to Erlotinib, which these cells were otherwise completely resistant to in our experiments. Finally, our novel compound was as potent as the reference VPS34 inhibitor and even more potent than the lysosomal inhibitor in its additive effects on cancer cell cytotoxicity. These results provide a sound basis for further preclinical and clinical studies of SB02024 and its combinations with Sunitinib or Erlotinib in the treatment of breast cancer, and thus strongly support VPS34 as a promising target in anti-cancer treatment.

4.4 PAPER IV

Regulation of RASAL2 and RASAL2-AS1 during autophagy.

Motivation

Autophagy is an essential pathway securing cellular homeostasis, especially under stress conditions. The delicate balance between catabolism and anabolism requires tight regulation. Recently, long-non-coding RNAs (lncRNA) have emerged next to transcriptional and translational regulation in the role of fine-tuning protein expression but also protein function (Kopp & Mendell 2018). In this study, we sought out to identify novel lncRNA and proteins, which are differentially expressed when autophagy is induced and to study their link to autophagy regulation.

Main findings

We have found that *RASAL2-AS1*, a primarily nuclear-located lncRNA, and its sense counterpart, mRNA of the protein-coding gene RASAL2, were induced upon autophagy activation, via mTOR inhibition either by small molecules or by amino-acid starvation. In contrast, RASAL2 protein levels were either marginally increased or even decreased under conditions of prolonged autophagy induction. This decrease could be partially rescued by the addition of lysosomal inhibitors, which block the late stage of autophagy. Moreover, a knockdown of the critical autophagy regulator ATG7 caused a massive accumulation of RASAL2 protein. These data reinforced the hypothesis that autophagy may degrade RASAL2. We could demonstrate that knock-down of *RASAL2-AS1* led to an increase of the *RASAL2* transcript and also of the protein levels suggesting a negative regulation of RASAL2 by *RASAL2-AS1*. Furthermore, we showed that knock-down of RASAL2 inhibited LC3-lipidation and puncta formation in the GFP-LC3 transfected cell line. Using the bioinformatical analysis, we have identified multiple LC3-interacting regions (LIR) domain in the *RASAL2* sequence, which could provide a starting point for functional studies to understand the role of RASAL2 in LC3 lipidation.

Discussion

Autophagy is regulated at different levels and by a large number of proteins organized in cascades in a well-orchestrated and structural fashion (Lorin et al. 2013). The lncRNA networks can regulate transcription of genes involved in cancer; lncRNA specific expression can be associated with therapy resistance, and this knowledge can help to understand the mechanistic processes in cells and to develop novel diagnostic and prognostic biomarkers (Grandér & Johnsson 2016). AS-RNA represent one class of lncRNA. Notably, up to 70% of mammalian protein-coding transcripts have antisense partners pointing at an essential role of these transcripts in gene regulation (Huang et al. 2015; Villegas & Zaphiropoulos 2015). Identifying differentially expressed AS-RNA can also lead to the identification of novel protein-coding genes involved in the crucial processes for cancer cells.

The function of RASAL2, on the other hand, is well-known: it is a RAS-GTPase activating protein, GAP, shown to inhibit a family of oncogenic RAS proteins, involved in cancer development and progression (J. Shen et al. 2013). The role of RASAL2 in autophagy has, to our knowledge, never been addressed. RASAL2 can interact with each of the four members of RAS subfamily, MRAS, KRAS, HRAS and NRAS (Szklarczyk et al. 2014), and as a RAS-GAP, it can be involved in the inhibition of their function. Activated RAS is known to induce signal pathways leading to cell growth, survival and differentiation and could lead to inhibition of autophagy via the PI3K-mTOR axis. However, RNAi mediated knockdown of *RASAL2* did not result in any noticeable changes of phosphorylated ERK, a downstream target of the RAS pathway, in one experiment that we have performed. Furthermore, mTOR was inhibited in our experiments either by KU-0063794 or through AA-starvation. Thus, we hypothesized that the involvement of RASAL2 in the autophagy induction is likely to be independent of its role in regulating RAS protein activity. On the other hand, the regulation of *RASAL2* by the *RASAL2-AS1* may have an impact on the RAS signaling, and this remains to be investigated.

The gradual upregulation of *RASAL2-AS1* and *RASAL2* RNA over time, when either KU or AA-starvation are activating autophagy, may indicate that their transcription is induced in the second phase response. In response to starvation, transcriptional induction of multiple autophagy genes like LC3B, GABARAPL1, ATG12, VPS34, Beclin-1 or ULK2 mediated by e.g., the transcription factor forkhead box transcription factor class O 3 (FoxO3), has been reported (He & Klionsky 2009). In our experiments, *RASAL2* and *RASAL2-AS1* were both upregulated by either mTOR inhibitors or by AA-starvation. Moreover, a pharmacological activator of mTOR inhibited expression of both transcripts further supporting their regulation by the transcription factors controlled by mTOR. There is, however, another aspect of

regulation of mTOR activity namely through its recruitment to the lysosome surface by the Rag small GTPase complex allowing the small GTPase Ras homolog enriched in brain (RHEB) to ultimately activate mTOR (J. Kim & E. Kim 2016). Previous reports describe the necessity of ATP hydrolysis by the vacuolar H(+)-adenosine triphosphatase ATPase (V-ATPase) for amino acids to promote such a mTORC1 translocation to the lysosomal membrane (Zoncu et al. 2011). In our experiments, however, blocking V-ATPase by BafA1 did not result in any changes of *RASAL2-AS1* and *RASAL2* transcription under either basal conditions or upon autophagy induction suggesting that localization of mTOR to the lysosomes may not be involved. This data suggested that RASAL2 protein might be degraded through an autophagic process. This observation was confirmed by strikingly elevated RASAL2 protein levels when we inhibited autophagy by RNAi with ATG7 and Vps34. Thus, we hypothesized that RASAL2 is induced by mTOR inhibition and is rapidly degraded by autophagy, suggesting the existence of a negative feedback mechanism between RASAL2 and autophagy.

RASAL2 mRNA expression can also be regulated by the lncRNA *RASAL2-AS1*, as we could show that a knockdown of *RASAL2-AS1* leads to increased *RASAL2* mRNA levels, under either basal conditions or upon autophagy induction. The mechanism of the AS-RNA-mediated RASAL2 mRNA expression was not addressed in this study. The *RASAL2-AS1* and *RASAL2* variant 2 lie nearby head to head and therefore do not show any overlap (Kent et al. 2002; Villegas & Zaphiropoulos 2015). Also, the subcellular localization of *RASAL2-AS1* to the nucleus may suggest that the mechanism of its action may be through epigenetic modifications, such as recruitment of repressors/epigenetic modifiers to the *RASAL2* promoter.

Interestingly, however, we did not observe any noticeable effect of the *RASAL2-AS1* knockdown on autophagy, in contrast to the *RASAL2* knockdown. The *RASAL2-AS1* knockdown, however, alleviated the autophagy-dependent RASAL2 degradation in AA-starved cells providing evidence that regulation by *RASAL2-AS1* can have apparent phenotypic effects on RASAL2 protein. The AS-RNA can provide a fine-tuned regulation of expression of genes, as we showed before (Johnsson et al. 2013), which might be hard to reveal considering a dynamic RASAL2 protein turn-over during autophagy induction. Thus, further studies are needed to confirm and elaborate on the role of AS-RNA-mediated RASAL2 regulation in autophagy as well as the mechanistic role of RASAL2 in the autophagic process.

In memoriam Dan Grandér

When I was applying for my dissertation (like always stretching deadlines, *ein bißchen*)
I thought I would have considered everything: The application, this bureaucratic monstrosity, spanning short of 160 pages was neatly printed out. The papers were present
(including ethical permits, retina scan of my left eye and elementary school report)
and I had a solid six hours to go.

I felt

the silver lining of relief dawning at the horizon.

But, as so often, things were a bit more complicated and I ended up biking over 25 km through a stormy night, negating complications.

This night ride will always stay in my mind. It led me through numerous neighborhoods of Stockholm and it was a little like a journey through time.
I thought about the pleasure I got from meeting so many people here...
Creative kinds, ambitious types, one smarter than the other!
And after a windy road I found myself close to where I first set foot coming from Uppsala 5 years ago, at Karlberg Station in front of Dannes apartment, full of melancholy and I wish, oh I wish so badly, he would come down the stairs, smile to me and say 'Slowly, but surely' one more time.

A FEW WORDS TO A FEW PEOPLE.

My supervisors:

First of all, **Katja**… my Doktormutter. I cannot thank you enough for your limitless support and encouragement. You have a heart of gold and a soul of a philosopher, combining seamlessly and effortlessly scientific rationale and empathy. Your humbleness is only being shadowed by your sharp mind, a combination every professor should have! Спасибо

Angelo, my brilliant co-supervisor, full of energy, thank you for your tremendous support; you are the incarnation of pubmed! It never fails to amaze me how your mind works; if an experiment fails your immediately see alternatives; instead of walls you see opportunities.

My moist friends:

Linda, the academic path and your own group is a given, I wish I would have only one tenth of your intellect and your humor. Thanks for keeping me up2date in Icelandic matters.

Elin, thanks for all the fun. I will miss your OCD and all the Nerf gun fights (and all the regular fights for that matter; it was always fun to discuss STUFF. See you in industry!

/dʒ/ aaaaaazmin,. You have such a great sunny side and esprit. Thanks for fantastic midsummers and just being such an amazing person. I would say 'Never change', but I know you won't! #RacistThursday. Am seeing you actually already in the industry.

Nathanael, Mr. **Andreeeeeews** thanks for all the AWs. You have a fantastic enthusiasm, never let anyone take it away! You just fit right in with your dry humor. PI #2.

Sander, miss you buddy. Thanks for a good time.

My colleagues:

Maaaaaaaartin, I really miss saying that. And listening to your computational flow dynamics of Gubbes Pasta á la Norma. I would have loved to have seen you together with Mikke doing ants experiments in the centrifuge.

Lotte, Thank you for your help and your kindness. It was a pleasure working with you in the group. You had always a smile on your lips!

Vasilios, I really hope we will manage to go for a run. I admire your clear headedness and your passion for even the littlest thing.

Jason, Yo!

My collaborators

Magda, thank you for your patience and for teaching me all there is about screening procedure. **Roland**, thank you for going 'meta' in metabolomics; it was lots of fun to discuss wild theories! **Paola** thank you for a good time either in the lab or on forest raves. **Florian**, thank you for having the best Core Facility at KI! Altijd leuk om ff bij te kletsen.

The Sprint connection:

Áine, Andreas, Jenny, Jessica, Santi, Tiago and **everyone at Sprint Bioscience**. It is great to see how *academic* industry can be. Thank you giving me a home on last stretch of my PhD. The spirit and energy in this company is truly unique.

Conference Call:

Many thanks to the Nov2k organization team: **Ahmed, Annika, Bettina, Elin, Kristina, Malte, Veronika** and of course **Karolina**. It was one of the highlights of my time at KI. And ofc to the various *From Basics to Clinics* orga teams; esp. to **Sebastian, Burcu, Carolin, Gonçalo, Helena, Sandra** and **Tina**! You guys rock! What a team effort!

Lords of Sci-Fi aka George's Brewery:

George, thanks for lots of psychology & philosophy bouldering sessions. You are an awesome dude; I can't stress this enough! **Wolfgäng**, the incredible result of crossing German Engineering with Australian Relaxation. It was a pleasure traveling Europe with you! **David G.**, du Kraftpacket, with a pinch of OCD… your unique serene view on the world is amazing. Stay as you are! **Michael**, Danish DIY PI and 3-dan of puns; missing your 'Fuck-it, let's do it' attitude. David S. Jetzt MACH doch EINFACH Digga! +++

CCK:

Alessandro, Bruce, Elin, Emarn, Matilda, My, Pedro, Sophia: You made this department easily a second home for me. Thanks for lots of fikas, pubs and seminars!

Andreas, Bertrand, Daniel, Jonas, Lars and **Mimi**: Thanks for your engagement in the department and lots of interesting chats!

Erika & Sören: Thank you for making my life so much easier!

The Ukrainian connection:

Greg, the only person I have first met in bed before being on the feet, my favorite ultra-running dentist and over all superman; **Dmitry**, the most passionate and still easy-going surgeon I know and **Mirjam**, never too shy to jump into the balkan beats crowd: Thanks for all the great dinners and concerts. I wish I would have been more there!

Susi, thank you for being my climbing partner; it was always fun die Mauer zu bezwingen. And thanks for all the CMM pubs! Shout out to **Eliane** ☺ Super gezellig.

Thank you, **Tiago**, for organizing soo much, **Simona** for being the first at the dance floor and again **Gonçalo** for being the voice of reason from time to time, when needed.

The **Maaslos** crew: It is fantastic to have you as friends… Every year we get a bit older, but everything stays the same with you; Auf Pfingsten! Isi!!!! My soulmate mit Bindungsangst. Ewiger Runningpartner, der Würme zum glühen bringt. Rockstar Lausi, die einfach mal macht! Und das mit einem Lachen auf den Lippen. Caro mit ihren unglaublichen Charme und Stil, ob PI oder Farmer… oder Pharma? ☺ Stella natürlich Pharma! Aber auch Vollzeit Bäcker, wie auch immer das zeittechnisch geht….miraTOR, Bob, Leo und Marcimarc….
VIELEN DANK euch allen!

Last but not least, thank you **Sofia** for always being there, in my best, but also my worst times, sharing passion for art, music and diving and 'just' being part of my life.
THANK YOU!

REFERENCES

Baslan, T. & Hicks, J., 2017. Unravelling biology and shifting paradigms in cancer with single-cell sequencing. *Nature Reviews Cancer*, 17(9), pp.557–569.

Batlle, E. & Clevers, H., 2017. Cancer stem cells revisited., 23(10), pp.1124–1134.

Boag, J.M. et al., 2006. Altered glucose metabolism in childhood pre-B acute lymphoblastic leukaemia. *Leukemia*, 20(10), pp.1731–1737.

Bozic, I. et al., 2010. Accumulation of driver and passenger mutations during tumor progression. *Proceedings of the National Academy of Sciences of the United States of America*, 107(43), pp.18545–18550.

Brown, E.J. et al., 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*, 369(6483), pp.756–758.

Buentke, E. et al., 2011. Glucocorticoid-induced cell death is mediated through reduced glucose metabolism in lymphoid leukemia cells. *Blood Cancer Journal*, 1(7), pp.e31–e31.

Button, R.W. et al., 2017. Accumulation of autophagosomes confers cytotoxicity. *The Journal of biological chemistry*, 292(33), pp.13599–13614.

Chan, L.N. et al., 2017. Metabolic gatekeeper function of B-lymphoid transcription factors. *Nature*, 542(7642), pp.479–483.

Cheong, H. et al., 2011. Ammonia-induced autophagy is independent of ULK1/ULK2 kinases. *Proceedings of the National Academy of Sciences of the United States of America*, 108(27), pp.11121–11126.

Choudhury, S. et al., 2013. Dissecting the pathways that destabilize mutant p53: the proteasome or autophagy? *Cell cycle (Georgetown, Tex.)*, 12(7), pp.1022–1029.

Cicchini, M. et al., 2014. Autophagy regulator BECN1 suppresses mammary tumorigenesis driven by WNT1 activation and following parity. *Autophagy*, 10(11), pp.2036–2052.

Codogno, P., Mehrpour, M. & Proikas-Cezanne, T., 2012. Canonical and non-canonical autophagy: variations on a common theme of self-eating?, 13(1), pp.7–12.

Cojoc, M. et al., 2015. A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. *Seminars in Cancer Biology*, 31, pp.16–27.

Connor, J., 2018. Toxic Exposures: Mustard Gas and the Health Consequences of World War II in the United Statesby Susan L. SmithSusan L. Smith, Toxic Exposures: Mustard Gas and the Health Consequences of World War II in the United States. New Brunswick, NJ: Rutgers University Press, 2017. xiii, 187 pp. $29.95 US (cloth). *Canadian Journal of History*, 53(2), pp.311–313.

Corbet, C. & Feron, O., 2017. Tumour acidosis: from the passenger to the driver's seat. *Nature Reviews Cancer*, 17(10), pp.577–593.

Coutinho, A.E. & Chapman, K.E., 2011. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Molecular and Cellular Endocrinology*, 335(1), pp.2–13.

Denton, D., Nicolson, S. & Kumar, S., 2012. Cell death by autophagy: facts and apparent artefacts. *Cell death and differentiation*, 19(1), pp.87–95.

DeVita, V.T. & Chu, E., 2008. A History of Cancer Chemotherapy. *Cancer research*, 68(21), pp.8643–8653.

Downing, J.R. et al., 2012. The Pediatric Cancer Genome Project. *Nature Genetics*, 44(6), pp.619–622.

Duran, A. et al., 2008. The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis. *Cancer cell*, 13(4), pp.343–354.

Eisenberg-Lerner, A. et al., 2009. Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell death and differentiation*, 16(7), pp.966–975.

Eng, C.H. et al., 2010. Ammonia Derived from Glutaminolysis Is a Diffusible Regulator of Autophagy. *Sci. Signal.*, 3(119), pp.ra31–ra31.

Fisher, R., Pusztai, L. & Swanton, C., 2013. Cancer heterogeneity: implications for targeted therapeutics. *British Journal of Cancer*, 108(3), pp.479–485.

Funderburk, S.F., Wang, Q.J. & Yue, Z., 2010. The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond. *Trends in cell biology*, 20(6), pp.355–362. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0962892410000053X.

Galluzzi, L. et al., 2015. Autophagy in malignant transformation and cancer progression. *The EMBO journal*, 34(7), pp.856–880.

Gewirtz, D.A., 2014. The four faces of autophagy: implications for cancer therapy. *Cancer research*, 74(3), pp.647–651.

Gillies, R.J., Robey, I. & Gatenby, R.A., 2008. Causes and consequences of increased glucose metabolism of cancers. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*, 49 Suppl 2(Suppl_2), pp.24S–42S.

Girardi, J.P., Pereira, L. & Bakovic, M., 2011. De novo synthesis of phospholipids is coupled with autophagosome formation. *Medical hypotheses*, 77(6), pp.1083–1087.

Gnant, M., Harbeck, N. & Thomssen, C., 2011. St. Gallen 2011: Summary of the Consensus Discussion. *Breast Care*, 6(2), pp.136–141.

Gong, C. et al., 2013. Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells. *Oncogene*, 32(18), pp.2261–72– 2272e.1–11.

Gozuacik, D. & Kimchi, A., 2007. Autophagy and cell death. *Current topics in developmental biology*, 78, pp.217–245. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0070215306780061.

Grandér, D. & Johnsson, P., 2016. Pseudogene-Expressed RNAs: Emerging Roles in Gene Regulation and Disease. *Current topics in microbiology and immunology*, 394(Chapter 442), pp.111–126.

Grandér, D. & Panaretakis, T., 2010. Autophagy: cancer therapy's friend or foe? *Future medicinal chemistry*, 2(2), pp.285–297.

Grandér, D. et al., 2009. Autophagy as the main means of cytotoxicity by glucocorticoids in hematological malignancies. *Autophagy*, 5(8), pp.1198–1200.

Grever, M.R., Schepartz, S.A. & Chabner, B.A., 1992. The National Cancer Institute: cancer drug discovery and development program. *Seminars in oncology*, 19(6), pp.622–638.

Guo, H. et al., 2014. Autophagy supports genomic stability by degrading retrotransposon RNA. *Nature communications*, 5, p.5276.

Hanahan, D. & Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell*, 144(5), pp.646–674.

He, C. & Klionsky, D.J., 2009. Regulation Mechanisms and Signaling Pathways of Autophagy. *Annual Review of Genetics*, 43(1), pp.67–93.

HEIDELBERGER, C. et al., 1957. Fluorinated Pyrimidines, A New Class of Tumour-Inhibitory Compounds. *Nature*, 179(4561), pp.663–666.

Holleman, A. et al., 2009. Gene-Expression Patterns in Drug-Resistant Acute Lymphoblastic Leukemia Cells and Response to Treatment. *dx.doi.org.proxy.kib.ki.se*, 351(6), pp.533–542.

Huang, S. et al., 2015. A new microRNA signal pathway regulated by long noncoding RNA TGFB2-OT1 in autophagy and inflammation of vascular endothelial cells. *Autophagy*, 11(12), pp.2172–2183.

Hulleman, E. et al., 2009. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. *Blood*, 113(9), pp.2014–2021.

Huss, M. & Wieczorek, H., 2009. Inhibitors of V-ATPases: old and new players. *Journal of Experimental Biology*, 212(3), pp.341–346.

Ignatiadis, M. & Sotiriou, C., 2013. Luminal breast cancer: from biology to treatment. *Nature Reviews Clinical Oncology*, 10(9), pp.494–506.

Inaba, H., Greaves, M. & Mullighan, C.G., 2013. Acute lymphoblastic leukaemia. *The Lancet*, 381(9881), pp.1943–1955.

Itakura, E., Kishi-Itakura, C. & Mizushima, N., 2012. The Hairpin-type Tail-Anchored SNARE Syntaxin 17 Targets to Autophagosomes for Fusion with Endosomes/Lysosomes. *Cell*, 151(6), pp.1256–1269.

Jaber, N. et al., 2012. Class III PI3K Vps34 plays an essential role in autophagy and in heart and liver function. *Proceedings of the National Academy of Sciences of the United States of America*, 109(6), pp.2003–2008.

Jackson, S.E. & Chester, J.D., 2014. Personalised cancer medicine. *Int J Cancer*, 137(2), pp.262–266.

Jangamreddy, J.R. et al., 2013. Salinomycin induces activation of autophagy, mitophagy and affects mitochondrial polarity: differences between primary and cancer cells. *Biochimica et biophysica acta*, 1833(9), pp.2057–2069.

Janku, F. et al., 2011. Autophagy as a target for anticancer therapy. *Nature Reviews Clinical Oncology*, 8(9), pp.528–539.

Jing, D. et al., 2014. Opposing regulation of BIM and BCL2 controls glucocorticoid-induced apoptosis of pediatric acute lymphoblastic leukemia cells. *Blood*, 125(2), pp.blood–2014–05–576470–283.

Johnsson, P. et al., 2013. A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells. *Nature structural & molecular biology*, 20(4), pp.440–446.

Jung, M. et al., 2015. Cathepsin inhibition-induced lysosomal dysfunction enhances pancreatic beta-cell apoptosis in high glucose. S. M. Srinivasula, ed. *PloS one*, 10(1), p.e0116972.

Karthik, G.-M. et al., 2017. Intra-tumor heterogeneity in breast cancer has limited impact on transcriptomic-based molecular profiling. *BMC cancer*, 17(1), p.802.

Kenific, C.M. & Debnath, J., 2015. Cellular and metabolic functions for autophagy in cancer cells. *Trends in cell biology*, 25(1), pp.37–45.

Kent, W.J. et al., 2002. The human genome browser at UCSC. *Genome Research*, 12(6), pp.996–1006.

Kopp, F. & Mendell, J.T., 2018. Functional Classification and Experimental Dissection of Long Noncoding RNAs. *Cell*, 172(3), pp.393–407.

Kreso, A. & Dick, J.E., 2014. Evolution of the Cancer Stem Cell Model. *Cell Stem Cell*, 14(3), pp.275–291.

Laane, E. et al., 2009. Cell death induced by dexamethasone in lymphoid leukemia is mediated through initiation of autophagy. *Cell death and differentiation*, 16(7), pp.1018–1029.

Lapierre, L.R. et al., 2015. Transcriptional and epigenetic regulation of autophagy in aging. *Autophagy*, 11(6), pp.867–880.

Lazova, R. et al., 2012. Punctate LC3B expression is a common feature of solid tumors and associated with proliferation, metastasis, and poor outcome. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 18(2), pp.370–379.

Lee, E.-J. & Tournier, C., 2011. The requirement of uncoordinated 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. *Autophagy*, 7(7), pp.689–695.

Levine, B. et al., 2015. Beclin orthologs: integrative hubs of cell signaling, membrane trafficking, and physiology. *Trends in cell biology*, 25(9), pp.533–544.

Li, Z. et al., 2016. Ammonia Induces Autophagy through Dopamine Receptor D3 and MTOR R. Kang, ed. *PloS one*, 11(4), p.e0153526.

Lorin, S. et al., 2013. Autophagy regulation and its role in cancer. *Seminars in Cancer Biology*, 23(5), pp.361–379.

Mai, T.T. et al., 2017. Salinomycin kills cancer stem cells by sequestering iron in lysosomes. *Nature chemistry*, 9(10), pp.1025–1033.

Managò, A. et al., 2015. Early effects of the antineoplastic agent salinomycin on mitochondrial function. *Cell Death & Disease*, 6(10), pp.e1930–e1930.

Marcucci, F., Ghezzi, P. & Rumio, C., 2017. The role of autophagy in the cross-talk between epithelial-mesenchymal transitioned tumor cells and cancer stem-like cells. *Molecular Cancer*, 16(1), p.3.

Marino, M.L. et al., 2012. Autophagy is a protective mechanism for human melanoma cells under acidic stress. *The Journal of biological chemistry*, 287(36), pp.30664–30676.

Martincorena, I. & Campbell, P.J., 2015. Somatic mutation in cancer and normal cells. *Science (New York, N.Y.)*, 349(6255), pp.1483–1489.

Mathew, R. et al., 2014. Functional role of autophagy-mediated proteome remodeling in cell survival signaling and innate immunity. *Molecular cell*, 55(6), pp.916–930.

Maycotte, P. et al., 2014. STAT3-mediated autophagy dependence identifies subtypes of breast cancer where autophagy inhibition can be efficacious. *Cancer research*, 74(9), pp.2579–2590.

Mazurek, S. & Shoshan, M., 2015. Tumor Cell Metabolism. p.374.

Meijer, A.J. et al., 2015. Regulation of autophagy by amino acids and MTOR-dependent signal transduction. *Amino acids*, 47(10), pp.2037–2063.

Meyer, L.H. et al., 2011. Early relapse in ALL is identified by time to leukemia in NOD/SCID

mice and is characterized by a gene signature involving survival pathways. *Cancer cell*, 19(2), pp.206–217.

Mizushima, N., 2018. A brief history of autophagy from cell biology to physiology and disease. *Nature cell biology*, 20(5), pp.521–527.

Mizushima, N. & Komatsu, M., 2011. Autophagy: renovation of cells and tissues. *Cell*, 147(4), pp.728–741.

Mizushima, N. et al., 2008. Autophagy fights disease through cellular self-digestion. *Nature*, 451(7182), pp.1069–1075.

Mizushima, N., Yoshimori, T. & Ohsumi, Y., 2011. The Role of Atg Proteins in Autophagosome Formation. *Annual Review of Cell and Developmental Biology*, 27(1), pp.107–132.

Mullighan, C.G. et al., 2008. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science (New York, N.Y.)*, 322(5906), pp.1377–1380.

Nagelkerke, A. et al., 2015. Therapeutic targeting of autophagy in cancer. Part II: pharmacological modulation of treatment-induced autophagy. *Seminars in Cancer Biology*, 31, pp.99–105. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1044579X14000765.

Namba, T. et al., 2014. Autophagic clearance of mitochondria in the kidney copes with metabolic acidosis. *Journal of the American Society of Nephrology : JASN*, 25(10), pp.2254–2266.

Neal, J.W. & Sledge, G.W., 2014. Decade in review-targeted therapy: successes, toxicities and challenges in solid tumours. *Nature Reviews Clinical Oncology*, 11(11), pp.627–628.

Ninomiya, T. et al., 2017. Iron depletion is a novel therapeutic strategy to target cancer stem cells. *Oncotarget*, 8(58), pp.98405–98416.

Novikoff, A.B. & Essner, E., 1962. Cytolysomes and mitochondrial degeneration. *The Journal of cell biology*.

Onorati, A.V. et al., 2018. Targeting autophagy in cancer. *Cancer*, 466, p.68.

Pan, H. et al., 2014. Autophagy inhibition sensitizes hepatocellular carcinoma to the multikinase inhibitor linifanib. *Scientific reports*, 4(1), p.6683.

Pasquier, B., 2015. Autophagy inhibitors. *Cellular and molecular life sciences : CMLS*, 73(5), pp.1–17.

Pellegrini, P. et al., 2014. Acidic extracellular pH neutralizes the autophagy-inhibiting activity of chloroquine. *Autophagy*, 10(4), pp.562–571. Available at: http://www.tandfonline.com/doi/abs/10.4161/auto.27901.

Petherick, K.J. et al., 2015. Pharmacological inhibition of ULK1 kinase blocks mammalian target of rapamycin (mTOR)-dependent autophagy. *The Journal of biological chemistry*, 290(48), pp.28726–28726.

Piao, S. et al., 2017. ALDH1A1 and HLTF modulate the activity of lysosomal autophagy inhibitors in cancer cells. *Autophagy*, 10, pp.1–16.

Plaks, V., Kong, N. & Werb, Z., 2015. The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell*, 16(3), pp.225–238.

Prat, A. et al., 2015. Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast (Edinburgh, Scotland)*, 24 Suppl 2, pp.S26–35.

Proikas-Cezanne, T. et al., 2015. WIPI proteins: essential PtdIns3P effectors at the nascent autophagosome. *Journal of cell science*, 128(2), pp.207–217.

Pui, C.-H. et al., 2015. Childhood Acute Lymphoblastic Leukemia: Progress Through Collaboration. *Journal of Clinical Oncology*, 33(27), pp.2938–2948.

Qadir, M.A. et al., 2008. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. *Breast Cancer Research and Treatment*, 112(3), pp.389–403.

Qiang, L. et al., 2014. Regulation of cell proliferation and migration by p62 through stabilization of Twist1. *Proceedings of the National Academy of Sciences of the United States of America*, 111(25), pp.9241–9246.

Rabinowitz, J.D. & White, E., 2010. Autophagy and metabolism. *Science (New York, N.Y.)*, 330(6009), pp.1344–1348.

Reggiori, F. & Klionsky, D.J., 2002. Autophagy in the eukaryotic cell. *Eukaryotic Cell*, 1(1), pp.11–21.

Rello-Varona, S. et al., 2012. Autophagic removal of micronuclei. *Cell cycle (Georgetown, Tex.)*, 11(1), pp.170–176.

Rogov, V. et al., 2014. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Molecular cell*, 53(2), pp.167–178.

Rose, A.J. & Herzig, S., 2013. Metabolic control through glucocorticoid hormones: An update. *Molecular and Cellular Endocrinology*, 380(1-2), pp.65–78.

Rouschop, K.M.A. et al., 2010. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *The Journal of clinical investigation*, 120(1), pp.127–141.

Saeed, K. et al., 2017. Comprehensive Drug Testing of Patient-derived Conditionally Reprogrammed Cells from Castration-resistant Prostate Cancer. *European urology*, 71(3), pp.319–327.

Saito, T. et al., 2016. p62/Sqstm1 promotes malignancy of HCV-positive hepatocellular carcinoma through Nrf2-dependent metabolic reprogramming. *Nature communications*, 7, p.12030.

Selvakumaran, M. et al., 2013. Autophagy Inhibition Sensitizes Colon Cancer Cells to Antiangiogenic and Cytotoxic Therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 19(11), pp.2995–3007.

Settembre, C. et al., 2011. TFEB Links Autophagy to Lysosomal Biogenesis. *Science (New York, N.Y.)*, p.1204592.

Shen, J., Wang, Y. & Hung, M.-C., 2013. RASAL2: wrestling in the combat of Ras activation. *Cancer cell*, 24(3), pp.277–279.

Shen, S. et al., 2011. Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. *Oncogene*, 30(45), pp.4544–4556.

Sorlie, T. et al., 2003. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences of the United States of America*, 100(14), pp.8418–8423.

Soto, A.M. & Sonnenschein, C., 2004. The somatic mutation theory of cancer: growing problems with the paradigm? *BioEssays : news and reviews in molecular, cellular and developmental biology*, 26(10), pp.1097–1107.

Stewart, B.W. & Wild, C.P., 2014. *World Cancer report 2014*

Suárez, I., Bodega, G. & Fernández, B., 2002. Glutamine synthetase in brain: effect of ammonia. *Neurochemistry International*, 41(2-3), pp.123–142.

Swerdlow, S. et al., 2008. Apoptosis inhibition by Bcl-2 gives way to autophagy in glucocorticoid-treated lymphocytes. *Autophagy*, 4(5), pp.612–620.

Szklarczyk, D. et al., 2014. STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, 43(D1), pp.D447–D452.

Takahashi, Y. et al., 2013. Bif-1 haploinsufficiency promotes chromosomal instability and accelerates Myc-driven lymphomagenesis via suppression of mitophagy. *Blood*, 121(9), pp.1622–1632.

Viale, G., 2012. The current state of breast cancer classification. *Annals of Oncology*, 23(suppl_10), pp.x207–x210.

Villegas, V.E. & Zaphiropoulos, P.G., 2015. Neighboring gene regulation by antisense long non-coding RNAs. *International journal of molecular sciences*, 16(2), pp.3251–3266.

Wang, Y. et al., 2013. Autophagy-dependent ATP release from dying cells via lysosomal exocytosis. *Autophagy*, 9(10), pp.1624–1625.

Wojtkowiak, J.W. et al., 2012. Chronic autophagy is a cellular adaptation to tumor acidic pH microenvironments. *Cancer research*, 72(16), pp.3938–3947.

Wolf, J. et al., 2013. A mammosphere formation RNAi screen reveals that ATG4A promotes a breast cancer stem-like phenotype. *Breast cancer research : BCR*, 15(6), p.R109.

Wu, S. et al., 2013. Biochemical and Biophysical Research Communications. *Biochemical and Biophysical Research Communications*, 434(4), pp.898–903.

Wu, Y.-T. et al., 2010. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *The Journal of biological chemistry*, 285(14), pp.10850–10861.

Yang, Y. et al., 2015. Autophagy and its function in radiosensitivity. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, 36(6), pp.4079–4087.

Young, A.R.J. et al., 2009. Autophagy mediates the mitotic senescence transition. *Genes & development*, 23(7), pp.798–803.

Zoncu, R. et al., 2011. mTORC1 Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacuolar H+-ATPase. *Science (New York, N.Y.)*, 334(6056), pp.678–683.